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Award Number: W81XWH-09-1-0640

TITLE: Functional Genomics for Epithelial-Mesenchymal Transition in Breast Cancer

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REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-10-2011	Annual	3 SEP 2010 - 2 SEP 2011

4. TITLE AND SUBTITLE
Functional Genomics for Epithelial-Mesenchymal Transition in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0640

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Melbourne
Parkville 3052

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

To assess whether modulation of the PMC42-LA cell line by a library of shRNAmir constructs can cause tumorigenicity, the impact of a smaller boutique library is first being assessed, as proposed. This boutique library of 4,462 shRNA constructs targeting 1,860 markers and mediators of EMT, metastasis, migration, and BCSC has been transduced into the PMC42-LA cell line as a total pool and as ten smaller sub-pools. These were expanded in preparation for in vivo testing. The smaller sub-pools have greater representation of individual shRNAmir constructs. For any sub-pool containing shRNAmir constructs that enables tumorigenicity, this greater representation translates to a markedly larger inoculum of tumorigenic cells, which may be important in the establishment of a tumor. The presence of a small but significant proportion of colonies exhibiting markedly mesenchymal features has been observed in the transduced pools, validating the use of this boutique library pool to directly assess whether PMC42-LA tumors can be grown using pools containing a proportion of more mesenchymal cells as an inoculum prior to assessing the whole genome shRNAmir library. Various methodologies for the in vitro selection of transduced cells with mesenchymal features from these pools, supplementary to the initial aims, are under development.

15. SUBJECT TERMS
Key words or phrases identifying major concepts in the report: Epithelial Mesenchymal Transition (EMT), functional genomics, micrometastasis, macrometastasis, shRNA

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	40	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

We hypothesize that epithelial – mesenchymal phenotypic attributes affect the capacity of single cells to establish a macroscopically detectable cancer mass, and thus play an etiological role in tumorigenicity, invasion, metastasis and recovery after seemingly effective chemotherapy of breast cancer cells. This will increase our understanding of the role of EMT in breast cancer, provide novel reagents and tools for the study of breast cancer, and provide new leads for therapeutic targeting in breast cancer. We are employing the high throughput functional genomic screens using epithelial mesenchymal transition (EMT)-capable PMC42 and MDA-MB-468 human breast cancer cell lines to identify molecular factors controlling these processes, and test their relationship to the EMT process. To date we have progressed the project with the PMC42-LA cells to the extent that tumors have been found after ~ 7 months. Parallel analysis in vitro has shown morphological changes and altered profile of cell surface EpCam, which we have independently validated to associate with increased EMT. Some of the year two tasks are behind schedule, however the project has accelerated with the recent observation of tumor growth and we anticipate making up considerable ground in the third year.

Body

Task 1: To identify gene products which may constitutively block the growth of PMC42 human breast cancer cells in SCID mice.
These cells undergo EGF-induced EMT and show BCSC attributes, but do not grow in SCID mice when implanted orthotopically or introduced intra-cardially.

1a **(months 1-3)** Luciferase introduction into PMC42-LA.
Deliverables: Luciferase-tagged cells for tracking of metastasis in vivo.

As PMC42-LA cells transduced with the boutique library and sub-pools thereof grew in the orthotopic model, but not the disseminated model, luciferase-tagging of these cells is not required for screening of the whole genome library.

1b **(months 3-6)** Pre-titration of shRNAmir library to determine optimal multiplicity of infection.
Deliverables: Optimal MOI for PMC42-LA cells

Completed in year 1.

1c **(months 6-9)** Transduction of luciferase-tagged PMC42-LA with boutique shRNAmir libraries, followed by expansion and enrichment of transduced cells.
Deliverables: Stocks of luciferase-tagged PMC42-LA cells, transduced with a boutique library comprising 3,600 shRNA clones targeting 1,746 genes selected as markers and mediators of EMT, metastasis, migration, and BCSC.

Completed in year 1 with untagged PMC42-LA cells, due to difficulties in tagging as described in the previous report. As mentioned above, the shRNA-transduced pools / subpools developed tumors in the orthotopic model, but not the disseminated model (intracardiac inoculation), obviating the need for tagged cells in going forward.

1d **(months 9-12)** Orthotopic and Disseminated growth of boutique shRNAmir transduced PMC42-LA cells.
Deliverables: Tumors in which a block in tumorigenicity has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

Mice were inoculated with PMC42-LA cells transduced with either the boutique shRNAmir library (~5,000 shRNA) or sub-pools (9 subpools of ~500 shRNA) of the same library. For any sub-pool containing shRNAmir constructs that enable tumorigenicity, this greater representation translates to a markedly larger inoculum of tumorigenic cells, which could have been important in the establishment of a tumor in this model.

In the orthotopic model, tumors grew by seven months in all mice that did not die earlier from unrelated health issues, with the earliest detected tumor at six months (Table 1).

Table 1

Group	mice that grew tumours/ mice that survived to 6 months
total boutique library	3/3
boutique sub-pool #1	3/3
boutique sub-pool #2	3/3
boutique sub-pool #3	3/3
boutique sub-pool #5	2/2
boutique sub-pool #6	3/3
boutique sub-pool #7	3/3
boutique sub-pool #8	3/3
boutique sub-pool #9	3/3
boutique sub-pool #10	3/3

Tumors have been collected for the identification of the shRNAmir constructs present in the cells that grew. The H&E staining of tumors indicated that they are semi- to well differentiated adenocarcinomas (Figure 1).

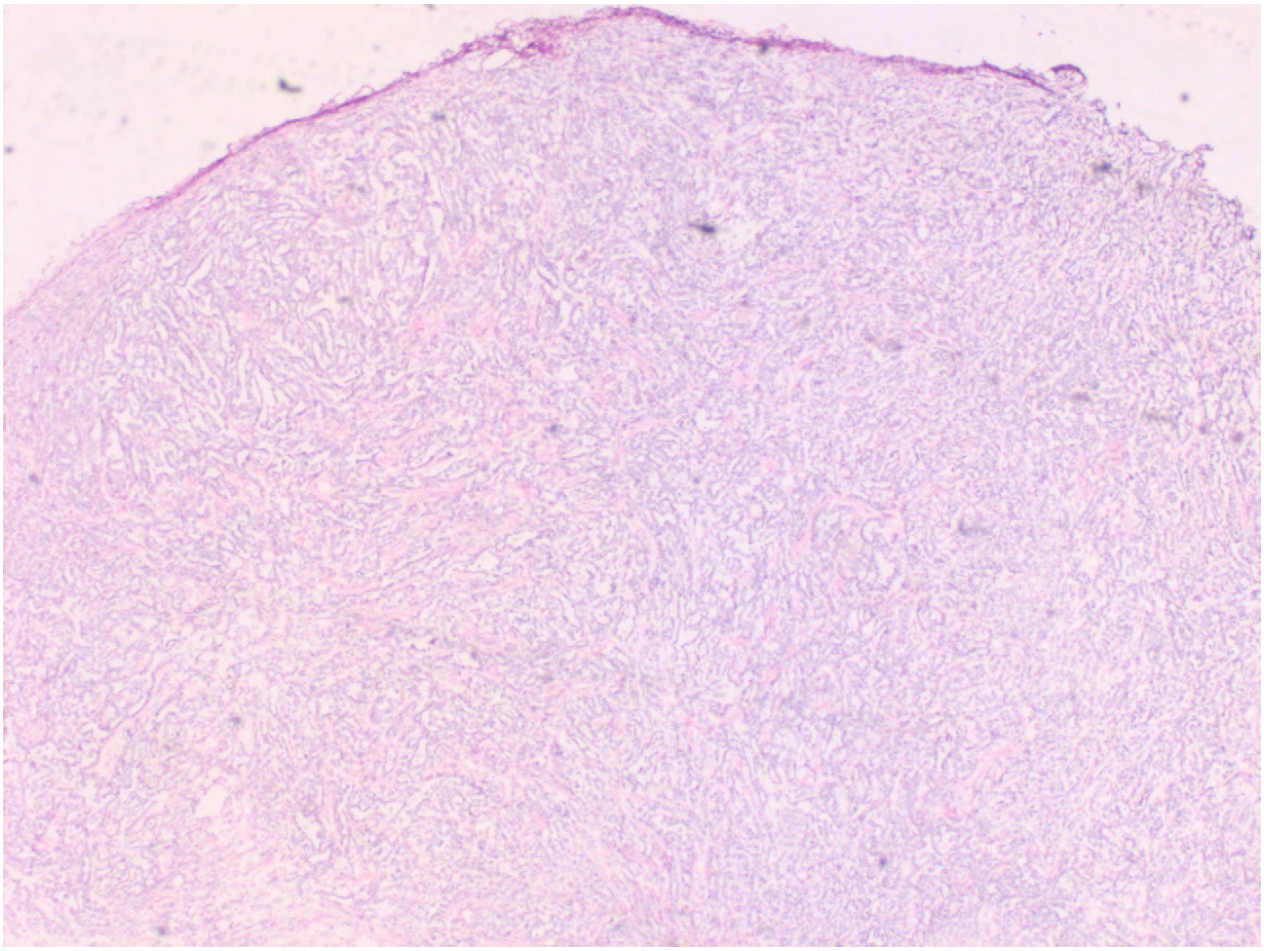

Figure 1: H&E staining representative of the tumors arising.

In the dissemination model, mice were monitored for eight months after the inoculation of PMC42-LA cells transduced with either the boutique shRNAmir library or sub-pools of the same library. No tumors were detected.

1e **(months 12-18)** Transduction of luciferase-tagged PMC42-LA with whole genome shRNAmir libraries, followed by expansion and enrichment of transduced cells.
Deliverables: Stocks of luciferase-tagged PMC42-LA cells, transduced with ~ 100,000 whole genome shRNA clones.

Transduction of PMC42-LA with whole genome shRNAmir libraries is currently awaiting validation of the approach after use of the boutique library, which will be determined by an assessment of whether there has been a specific enrichment for various shRNAmir constructs in the tumors compared to the cell pools that were initially injected into the mice.

1f **(months 9-12)** Orthotopic **or** Disseminated growth of whole genome shRNAmir transduced PMC42-LA cells.
Deliverables: Tumors in which a block in tumorigenicity has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

As a result of the development of tumors by boutique shRNAmir transduced PMC42-LA cells only in the orthotopic model, this model has been selected in order to screen the whole genome shRNAmir library.

Task 2: To identify gene products which may constitutively block the spontaneous capacity of MDAMB-468 human breast cancer cells to form metastases in SCID mice.
These cells undergo EMT at the xenograft edge and liberate CTCs, but form micrometastases at low frequency. They do not form overt lesions when inoculated intracardially. After genome-wide shRNA knockdown they will be screened for disseminated growth following intra-cardiac seeding, and distant metastasis following orthotopic inoculation.

2a **(months 1-3)** Luciferase introduction into MDA-MB-468.
Deliverables: Luciferase-tagged MDA-MB-468 cells for tracking of metastasis in vivo.

MDA-MB-468 cells have been successfully transfected with pGL4.50[luc2/CMV/Hygro].

2b **(months 3-6)** Pre-titration of shRNAmir library to determine optimal multiplicity of infection.
Deliverables: Optimal MOI for MDA-MB-468 cells

A viral supernatant with a known viral titre has been re-titrated in the MDA-MB-468 cell line, alongside the HEK-293T cells, in which the viral titre was initially determined, enabling the viral dose required to give the optimal MOI to be determined. From the comparative titration it was determined that the MDA-MB-468 cell line is as efficiently transduced as the HEK-293T cells. It is worth noting that this is approximately 300-fold more effective than in the PMC42-LA cells, which appear quite refractory to all forms of transfection / transduction.

2c **(months 6-9)** Transduction of luciferase-tagged MDA-MB-468 with boutique shRNAmir libraries, followed by expansion and enrichment of transduced cells.
Deliverables: Stocks of luciferase-tagged MDA-MB-468 cells, transduced with a boutique library comprising 3,600 shRNA clones targeting 1,746 genes selected as markers and mediators of EMT, metastasis, migration, and BCSC.

As indicated in our last report, Tasks 2c and 2d were added to the SOW in error, as they are neither set out in the narrative nor covered in the budget. However, we do feel that it would be advantageous to have the boutique library arm included and we will look to include this within the current budget and with appropriate modification of the mouse numbers in the animal ethics. Please note that since we do hope to include this work, we have not submitted a modified SOW to remove it at this stage, as suggested by the reviewer of our first annual report.

2d **(months 9-12)** Disseminated growth of boutique shRNAmir transduced MDA-MB-468 cells.
Deliverables: Tumors in which a block in macrometastasis has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

Same as remarks regarding task 2c.

2e **(months 12-18)** Transduction of luciferase-tagged MDA-MB-468 with whole genome shRNAmir libraries, followed by expansion and enrichment of transduced cells.
Deliverables: Stocks of luciferase-tagged MDA-MB-468 cells, transduced with ~ 100,000 whole genome shRNA clones.

Transduction of the MDA-MB-468 cell line with whole genome shRNAmir libraries is currently awaiting validation of the approach by deep sequencing of the shRNAmirs present in the tumors that grew from PMC42-LA cells transduced with the boutique shRNAmir library in the orthotopic model compared with the initial cell pools that were injected into the mice.

2f **(months 19-24)** Disseminated growth of whole genome shRNAmir transduced MDA-MB-468 cells.
Deliverables: Tumors in which a block in macrometastasis has been overcome, enabling barcode analysis to determine the targeted genes. Collection of material for later identification of shRNAs.

Currently awaiting transduction of the MDA-MB-468 cell line with the whole genome shRNAmir libraries (see task 2e).

Task 3: To identify and characterise novel gene products elucidated in Aims 1 and 2 that enact a switch between epithelial and mesenchymal states.
Candidate shRNAs identified in Aims 1 and 2 will be assembled individually or assembled into a smaller, dedicated boutique screen and tested for their ability to influence EMT or MET. Novel candidates confirmed to play a role in EMT will be tested fully with *in vitro* and *in vivo* analysis.

3a **(months 1-12)** Transduction of various human breast cancer cell lines with epithelial (E-Cad-RFP) or mesenchymal (Vimentin-RFP) reporter constructs, and validation with known EMT perturbations.
Deliverables: Cells lines which faithfully report EMT and MET shifts.

MDA-MB-468, MCF-7, PMC42-LA and PMC42-ET cell lines have been stably transfected with epithelial (CDH1-BFP) or mesenchymal (Vimentin-RFP) reporter constructs. These transfected cells were assessed for reporter responsive populations through FACS analysis of EGF-treated and –untreated cells. MDA-MB-468 and MCF-7 cells transfected with the mesenchymal reporter construct demonstrated a decrease in the proportion of cells with low reporter activity following EGF treatment, indicating the presence of responsive subpopulations. Similarly, MDA-MB-468 and PMC42-LA cells transfected with the epithelial reporter construct demonstrated an increase in the proportion of cells with low reporter activity following EGF treatment, also indicating the presence of responsive subpopulations (Figure 2). These populations are undergoing further FACS enrichment to hone in on the most responsive subset. Combined co-transfection of both vectors may assist in this and is planned.

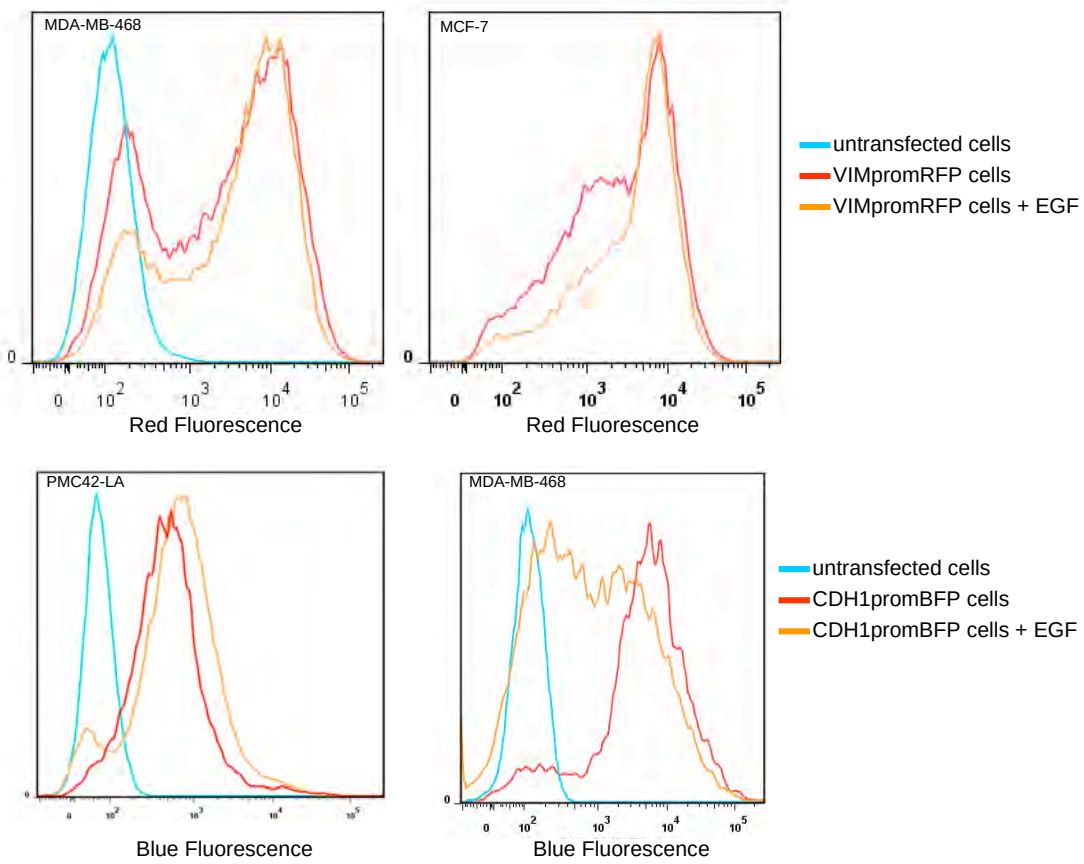

Figure 2: FACS analysis of VIM-RFP and E-Cad-BFP reporter expression in each of the indicated cell lines with / without treatment with EGF, compared to wild-type cells.

3b **(months 18-24)** Deep sequencing of assembled DNA barcodes / hairpins pooled from colonies arising in both boutique and whole genome screens in Tasks 1 and 2 **.**
Deliverables: Sequence identification of shRNAs present in colonies escaping repression of tumorigenesis and/or macrometastasis from Aims 1 and 2 respectively.

Deep sequencing of hairpins from tumors and the boutique total and sub-pools from which they grew is scheduled to be carried out at the VCFG as proposed. For sub-pools and the tumors that grew from them, a strategy involving indexing and deconvolution based upon which shRNAmir constructs are within each sub-pool will be used in order to minimise the number of sequencing lanes required.

As reported previously, mesenchymal colonies were observed in varying proportions when the PMC42-LA cells transduced with the boutique shRNAmir library sub-pools were plated at low density. These analyses are additional to those originally proposed but are consistent with the in vivo approach and we anticipate that analysis of the in vitro phenotype can be carried out in parallel. FACS analysis of wild type and library sub-pool transduced PMC42-LA cells for the epithelial marker EPCAM revealed that sub-pool #5, which exhibits the lowest proportion of mesenchymal colonies amongst the sub-pools, had a similar EPCAM profile to untransduced PMC42-LA cells, while sub-pool #7, which exhibits the largest proportion of mesenchymal colonies, had a markedly increased proportion of cells with low EPCAM expression (Figure 3).

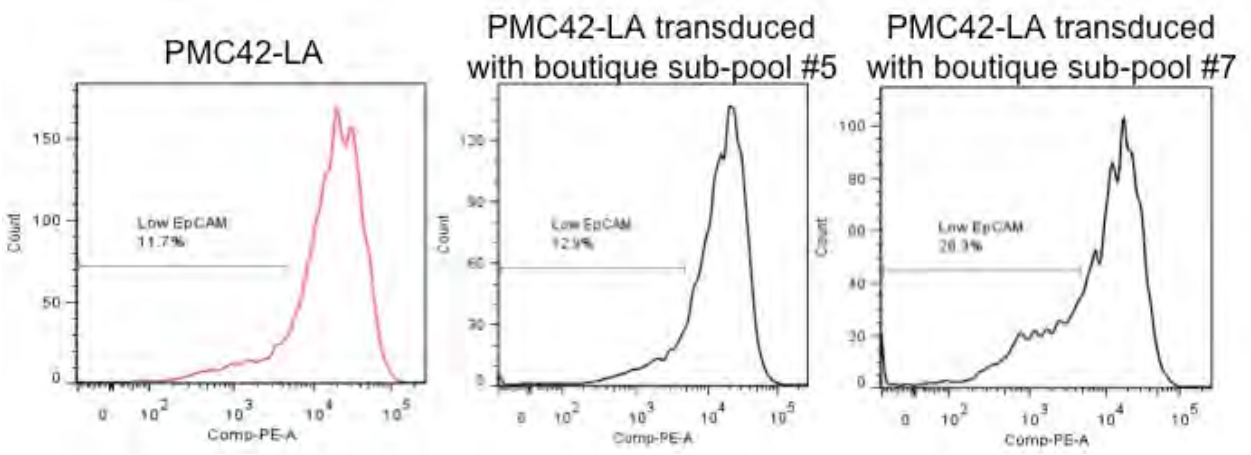

Figure 3: FACS analysis of EpCAM surface expression in untransduced PMC42-LA cells and selected subpools.

Morphologically PMC42-LA cells selected by FACS for high EPCAM expression are the same as the parental population (Figure 4a), whereas cells selected for low EPCAM expression predominantly exhibit mesenchymal features (Figure 4b).

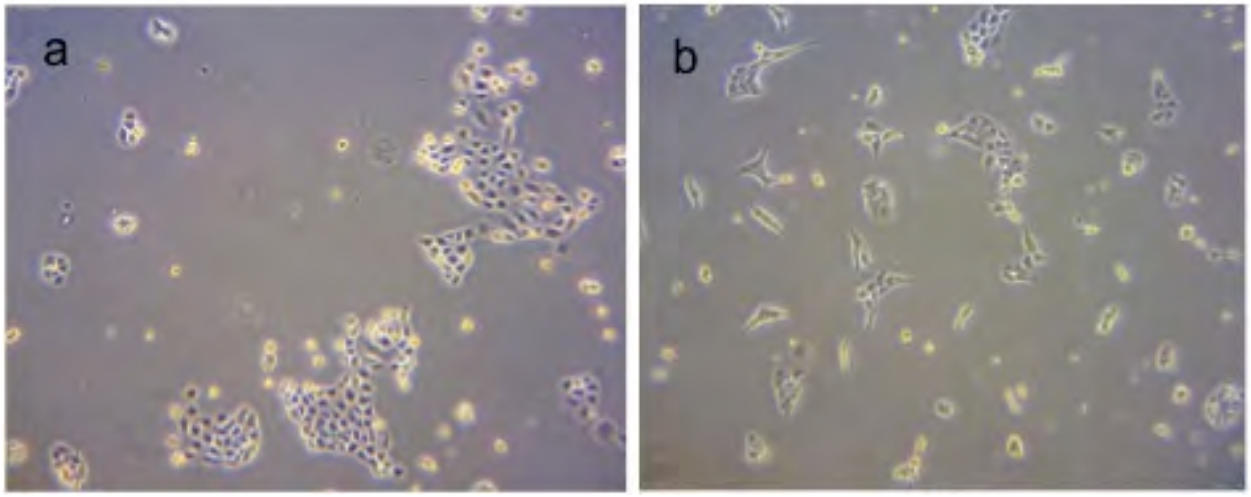

Figure 4: Culture morphology of PMC42-LA cells selected by FACS for high (a) and low (b) surface expression of EpCAM.

Analysis of EMT markers in these EpCAM-selected PMC42-LA cells by immunocytochemistry in comparison with the strongly mesenchymal MDA-MB-231 cells revealed

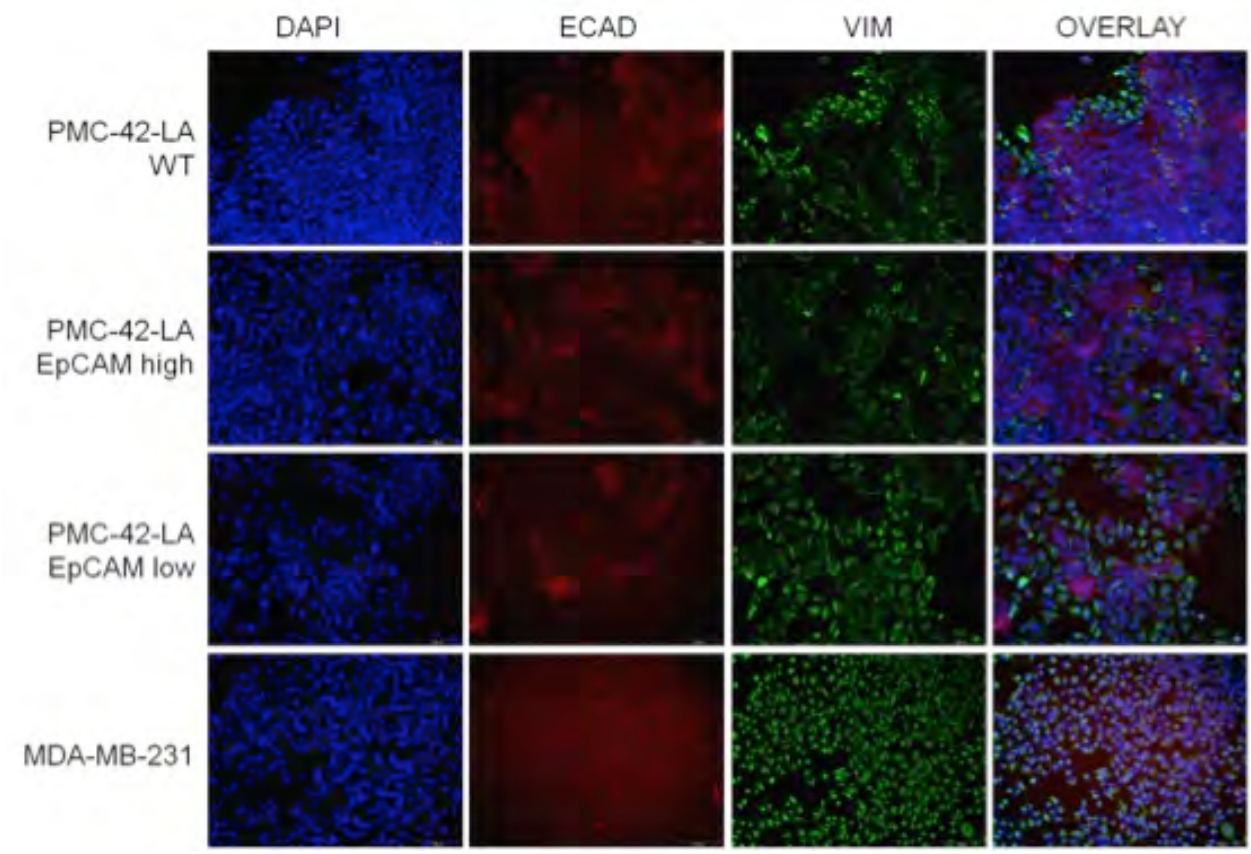

Figure 4: Immunocytochemical analysis of the EMT markers E-Cadherin (ECAD) and vimentin (VIM) in the unselected PMC42-LA (WT) cells, PMC42-LA cells selected for high or low EpCAM expression, and MDA-MB-231 cells.

Selection of the EPCAM-low sub-populations of the boutique library transduced PMC42-LA cells are under-way and the hairpins will be subjected to NGS, in order to ascertain which if any shRNAmirs are causative of this mesechymalisation.

3c **(months 24-27)** Screen boutique shRNA library with FACS-based, in-cell EMT/MET reporter assays
Deliverables: Identification of shRNA candidates with EMT perturbation potential.

Not covered prior to year 3

3d **(months 24-36)** Designation of EMT/MET/BCSC and non-EMT/MET/BCSC candidates.
Deliverables: Prioritization of EMT/MET/BCSC for this study, dissemination of non-EMT/MET/BCSC to the research community.

Not covered prior to year 3

3e **(months 27-36)** In vitro analysis of up to 10 EMT/MET perturbational candidates.
Deliverables: Characterization of the in vitro proliferative, migratory, and invasion-regulating potential of candidates, analysis of EMT perturbational mechanism, analysis of relationship to BCSC phenotype.

Not covered prior to year 3

3f **(months 30-36)** In vivo analysis of up to 3 EMT/MET perturbational candidates for effects on tumorigenic potential of PMC42-LA and macrometastatic potential of MDA-MB-468; analysis of molecular consequences and morphologic effects in vivo.
Deliverables: Characterization of the in vivo tumorigenic and macrometastatic potential of candidates, analysis of EMT perturbational mechanism, analysis of relationship to BCSC phenotype.

Not covered prior to year 3

3g **(months 24-36)** Manuscript preparation and submission, grant preparation.
Deliverables: Submitted grant applications for further work, dissemination of findings through conferences and publications.

Not covered prior to year 3

Key Research Accomplishments

- Orthotopic tumors have been grown from PMC42-LA cells transduced with a boutique library, and sub-pools thereof, consisting of shRNAmirs to targets selected as markers and mediators of EMT, metastasis, migration, and breast cancer stem cells. These tumors allow an assessment of which, if any hairpins, have enabled tumorigenicity.

- Responsive subpopulations have been detected in both MDA-MB-468 and MCF-7 cells transfected with a mesenchymal reporter construct, and both MDA-MB-468 and PMC42-LA cells transfected with an epithelial reporter construct. Following selection, these responsive subpopulations will enable measurements of epithelial and mesenchymal promoter states in the presence of the GFP-encoding GIPZ shRNA constructs.

- PMC42-LA cells transduced with the boutique library, and sub-pools thereof, have been determined to have altered proportions of cells capable of forming mesenchymal colonies. Sub-pools of transduced cells with the highest proportion of cells capable of forming mesenchymal colonies have also been found to have a larger proportion of cells with low EPCAM surface expression. Consistent with this, cells with low EPCAM surface levels have a more mesenchymal phenotype. This will enable selection of cells within the transduced sub-pools that are more mesenchymal and a determination of which if any shRNAmir constructs are enriched for in these subpopulations.

Reportable Outcomes

PRIMARY RESEARCH ARTICLES

EWT#128 Chand, A.L., Herridge, K.A., THOMPSON, E.W., Clyne, C.D. The orphan nuclear receptor LRH-1 promotes breast cancer motility and invasion. Endocrine-Related Cancer 17: 965-975 (2010) http://dx.doi.org/10.1677/ERC-10-0179. 'Faculty of 1000' article.

REVIEWS / **BOOK CHAPTERS**

EWT#BC15 Soon, L, Tachtsidis, A, Fok, S, Williams, ED, Newgreen, DF, THOMPSON, EW. The continuum of epithelial mesenchymal transition – implication of hybrid states for migration and survival in development and cancer. In Cancer Metastasis: Biologic Basis and Therapeutics. D Lyden, DR Welch and B Psaila eds. Cambridge University Press. Chapter 11, pp. 117-130 (2011) (ISBN-13: 9780521887212)

CONFERENCES PRESENTATIONS (Podium)

International

International Bone & Mineral Society – Cancer and Bone Society meeting, Chicago, IL, USA; Nov. 29 – Dec. 3, 2011. Epithelial Mesenchymal Plasticity and Breast Cancer Metastasis in Bone and Bone Marrow.

Joint TuMIC - Metastasis Research Society - Champalimaud Foundation Conference 'New Concepts in Cancer Metastasis', Champalimaud Cancer Centre, Lisbon, Portugal; June 25 – 28, 2011. Epithelial Mesenchymal Plasticity and Breast Cancer Metastasis – Insights from Human Breast Cancer Cell Lines. Also Session Chair, Session 8.

Keystone Symposium on Epithelial Plasticity and Epithelial to Mesenchymal Transition, Vancouver, B.C., Canada; Jan. 21-26, 2011. Epithelial Mesenchymal Plasticity in Human Breast Cancer Cell Lines: The Path Forward?

National

ANZ BCTG 33rd Annual Participants' Scientific Meeting, Royal Pines Resort, Gold Coast, QLD; July 22-23, 2011. Epithelial Mesenchymal Plasticity – New Opportunities for Targeting Breast Cancer.

The 16th International Colloquium on Lung and Airway Fibrosis, Busselton, WA; 30 October – 3 November, 2010. Epithelial Mesenchymal Plasticity and Pathogenesis: Breast Cancer as a Model Case.

SEMINAR PRESENTATIONS

International

Idibell, Barcelona, Spain (Host Prof. Angels Fabra-Fres); July 1, 2011. Multiple modes of regulation of Epithelial Mesenchymal Plasticity in the PMC42 human breast cancer model system.

National

Deakin University, School of Medicine (Host Dr Nicky Konstantopoulos); May 20, 2011. Epithelial Mesenchymal Plasticity, Mammographic Density and Matrix Metalloproteinase 13 in Breast Cancer.

St. Vincent's Hospital (Melbourne; Host Dr Tony Dowling), Breast cancer multidisciplinary meeting; March 29, 2011 The EMP*athy* Breast Cancer Network.

CONFERENCES PRESENTATIONS (Poster)

International

US-DOD Era of Hope, Orlando, Florida. August, 2011. Izhak Haviv, Tony Blick, Cletus Pinto, Mark Waltham, Erik Thompson. A Functional Genomic Screen for Tumorigenicity and Epithelial-Mesenchymal Transition

102nd Annual AACR meeting, Orlando, FLA, USA; April 2 – 6, 2011. Honor Hugo, Bryce JW van Denderen, Eva Tomaskovic-Crook, Tony Blick, Dexing Huang, Cletus Pinto, Eliza Soo, Angels Fabra-Fres, Izhak Haviv, Gregory Goodall, Nicholas Wong, Leigh Ackland, Donald F. Newgreen, Mark Waltham, Erik W. Thompson. Coordinated Regulation of Mesenchymal Epithelial Transition in the PMC42-LA Breast Cancer Cell Line Variant.

National
2012 Lorne Genome Conference, Lorne, Australia, February, 2011. Cletus Pinto, Tony Blick, Izhak Haviv, Mark Waltham, Erik Thompson. Understanding Epithelial Mesenchymal Plasticity in Breast Cancer – A Functional Genomics Approach

CONCLUSION

Although yet to be proven, the results to date with the boutique shRNAmir library indicate that we have succeeded in generating mesencymally-shifted PMC42-LA cells, and that these populations show enhanced tumorigenicity.

The orphan nuclear receptor LRH-1 promotes breast cancer motility and invasion

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*(A L Chand and K A Herridge contributed equally to this work)

Abstract

The orphan nuclear receptor liver receptor homologue-1 (LRH-1) has roles in the development, cholesterol and bile acid homeostasis, and steroidogenesis. It also enhances proliferation and cell cycle progression of cancer cells. In breast cancer, LRH-1 expression is associated with invasive breast cancer; positively correlates with ERα status and aromatase activity; and promotes oestrogen-dependent cell proliferation. However, the mechanism of action of LRH-1 in breast cancer epithelial cells is still not clear. By silencing or over-expressing LRH-1 in ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells, we have demonstrated that LRH-1 promotes motility and cell invasiveness. Similar effects were observed in the non-tumourigenic mammary epithelial cell line, MCF-10A. Remodelling of the actin cytoskeleton and E-cadherin cleavage was observed with LRH-1 over-expression, contributing to increased migratory and invasive properties. Additionally, in LRH-1 over-expressing cells, the truncation of the 120 kDa E-cadherin to the inactive 97 kDa form was observed. These post-translational modifications in E-cadherin may be associated with LRH-1-dependent changes to matrix metalloproteinase 9 expression. These findings suggest a new role of LRH-1 in promoting migration and invasion in breast cancer, independent of oestrogen sensitivity. Therefore, LRH-1 may represent a new target for breast cancer therapeutics.

Endocrine-Related Cancer (2010) **17** 965–975

Introduction

Liver receptor homologue-1 (LRH-1, NR5A2) belongs to the NR5A orphan nuclear receptor subfamily and has important roles in embryonic and adult tissues. LRH-1 expression in embryonic stem cells (ESC) allows activation of Oct 4, a requirement for early embryonic differentiation of primitive endoderm/mesoderm or trophectoderm (Gu *et al*. 2005). Other LRH-1 target genes important for endodermal differentiation include *GATA*, *Nkx*, *HNF3-β*, *HNF4-α* and *HNF1-α* (Pare *et al*. 2001). Consistent with its expression and function in embryonic tissue, the deletion of LRH-1 results in embryonic lethality (Galarneau *et al*. 1996, Gu *et al*. 2005).

In the adult tissue, LRH-1 has known functions in steroidogenesis, cholesterol homeostasis and carcinogenesis (reviewed in Francis *et al*. (2003), Clyne *et al*. (2004), Fayard *et al*. (2004) and Lee & Moore (2008)). LRH-1 has been shown to be a tumour-promoting transcription factor in colon, gastric and breast cancers (Botrugno *et al*. 2004, Schoonjans *et al*. 2005, Miki *et al*. 2006, Wang *et al*. 2008). LRH-1 increases cell proliferation in murine pancreatic LTPA and hepatic FL83B cells through the induction of cyclins, D1 and E1, with co-operative recruitment of β-catenin and LRH-1 to their promoters (Botrugno *et al*. 2004). In a mouse model of azoxymethane-induced colon neoplasia, a reduction in LRH-1 expression resulted

DOI: 10.1677/ERC-10-0179
1351–0088/10/017–965
Online version via http://www.endocrinology-journals.org

in reduced cancer incidence and better survival rates (Schoonjans *et al*. 2005). LRH-1 regulates intestinal crypt length and crypt cell proliferation through the G1 cyclins and c-Myc (Botrugno *et al*. 2004).

In pre- and post-menopausal breast cancer patients, high LRH-1 protein expression is detected in invasive ductal carcinomas (43%), ductal carcinoma *in situ* (28%) and adipose stromal cells (Annicotte *et al*. 2005, Zhou *et al*. 2005, Miki *et al*. 2006). This aberrant expression of LRH-1 in breast cancer stroma activates aromatase transcription thus increasing local oestrogen production within the tumour microenvironment (Bulun & Simpson 1994, Simpson & Davis 2001, Simpson *et al*. 2001, Zhou *et al*. 2005). This is a particularly relevant mechanism in post-menopausal breast tumours as intra-tumoural oestrogen levels and aromatase activity are high compared to pre-menopausal plasma and tissue levels (Pasqualini *et al*. 1996). *LRH-1* has also been described as an oestrogen target gene and shown to be a downstream effector of oestrogen-mediated cell proliferation (Annicotte *et al*. 2005). Hence, a positive feedback mechanism between LRH-1 and oestrogen production may be a critical component of oestrogen-dependent tumour growth. However, the question remains as to whether LRH-1 has additional roles, aside from oestrogen production in stromal cells, and whether LRH-1 plays a role in oestrogen-independent cancer growth.

LRH-1 is held in a constitutively active structural conformation with a stable fourth helical layer. Hence, its activity is modulated by the interaction with other co-activators and co-repressors (Goodwin *et al*. 2000, Lee & Moore 2002, Xu *et al*. 2004, Ortlund *et al*. 2005). Additionally, the identification of phospholipids (Krylova *et al*. 2005, Ortlund *et al*. 2005, Wang *et al*. 2005*b*) and sphingosine-1-phosphate (Hadizadeh *et al*. 2008) as LRH-1 agonists suggests that the degree of receptor activation may be further regulated with endogenous ligands. This also raises the possibility of pharmacological modulation of LRH-1 activity as treatment for intestinal, gastric and breast cancers. Since LRH-1 controls oestrogen production in a tissue-specific manner, potential LRH-1 antagonists could allow breast-specific suppression of aromatase activity (Safi *et al*. 2005).

Given the recent demonstrations of LRH-1 expression in breast carcinoma cells, this study tests the hypothesis that LRH-1 directly regulates breast cancer migration and invasion. We have found that LRH-1 directly regulates these processes by actin remodelling and post-translational inactivation of E-cadherin, consistent with the epithelial–mesenchymal transition (EMT). This effect also occurs independent of oestrogen and in non-cancerous cells such as MCF-10A. Based on these findings, LRH-1 is a potential candidate for the development of new breast cancer treatments.

Materials and methods

Plasmids, cell lines and transfections

Human LRH-1 pcDNA expression construct was generated by cloning into the pCDNA3.1 vector. The SureSilencing short hairpin RNA (shRNA) plasmids (KH05888G, SABiosciences, Frederick, MD, USA) were designed to specifically knockdown the expression of LRH-1 by RNA interference. Cells were transfected with the following plasmids pcDNA (control), pcDNA–LRH-1 (+LRH-1), scrambled shRNA (shcontrol) or shRNA against LRH-1 (shLRH-1). The following cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) MCF-7 (ATCC No. HTB-22), MCF-10A (ATCC No. CRL-10317) and MDA-MB-231 (ATCC No. HTB-26) and were cultured according to the manufacturer's procedures at 37 °C with 5% CO_2. Cells were transfected using electroporation (Nucleofector kit V, Amaxa Biosystems, Lonza, Walkersville, MD, USA), Solution V and the recommended programmes as follows: MCF-7 cells – P-20 (high transfection efficiency) and E-14 (high cell viability and short-term expression); MDA-MB-231 cells – X-013; and MCF-10A cells – T-024. These are described online (http://www.amaxa.com/research-areas/cancer/). Highest transfection efficiency is detected at 24–48 h post transfection and this was measured by GFP protein expression as expressed by the plasmid constructs, mRNA quantitation by qPCR and western blot analysis.

RNA extraction and quantitation of mRNA expression

Total RNA was extracted using the RNeasy Minikit (Qiagen) as described in the manufacturer's instructions. Reverse transcription was performed as instructed for 1 μg of RNA using the AMV Reverse Transcription System (Promega). qPCR amplification was performed on the Roche LightCycler System (Roche Diagnostics) using Fast Start Master SYBR Green 1 and specific primer sets (Sigma) as previously described for LRH-1 and 18S (Clyne *et al*. 2002). Primer sequences are as follows: LRH-1 (sense, CTG ATA CTG GAA CTT TTG AA; antisense, CTT CAT TTG GTC ATC AAC CTT); 18S (sense, CGG CTA CCA CAT CCA AGG A; antisense, GCT GGA ATT ACC GCG GCT);

E-cadherin (sense, GGC ACA GAT GGT GTG ATT ACA GTC AAA A; antisense, GTC CCA GGC GTA GAC CAA GAA A) and matrix metalloproteinase 9 (MMP9; sense, CGC TAC CAC CTC GAA CTT TG; antisense, GCC ATT CAC GTC GTC CTT AT). Experimental samples were repeated in triplicates for each transfection and quantified by comparison with a six-point standard curve as previously described (Chand *et al*. 2007).

Wound-healing migration assay

Transfected cells were serum-starved overnight before a scratch wound was created in the mono-confluent cell layer created with a sterile plastic pipette tip. Phase contrast microscopy images of the wound were taken at 0, 6, 12 and 24 h of the assay and gap closure measured using Analysis LS Professional (Soft Imaging Systems GmbH, Münster, Germany). For the accuracy of scratch/wound width, three scratch wounds were made per well and each transfection condition was repeated 3–4 times per experiment. Experiments were repeated at least 4 times.

Cytoskeleton F-actin staining with phalloidin

Transfected cells were allowed to attach to the coverslips before creation of a scratch wound in the confluent monolayer as described above. At 48 h post wound creation, cells were washed twice with PBS, fixed in 3.7% paraformaldehyde in PBS and permeabilised in 0.1% Triton X-100 and incubated with 200 μl of Alexa Fluor 488 phalloidin (Invitrogen) staining solution diluted in 1% BSA for 20 min at room temperature. Coverslips were then incubated with the nuclear stain TO-PRO-3 (Invitrogen) at a 1:200 dilution (PBS) covered for 10 min at room temperature. Following two PBS washes, the coverslips were mounted with fluorescent mounting media (Dako, Glostrup, Denmark) and were allowed to dry before being analysed. Immunofluorescence was visualised using an Olympus FV300 confocal microscope and images were captured and analysed using Olympus FluoViewTM software. Actin projections were counted in ten cells from each transfection condition and in three separate transfection experiments. Cell volume was measured using the ImageJ software (http://rsbweb.nih.gov/ij/).

Cell invasion assay

Following transfection, cells were serum-starved overnight. Cells were then plated at a density of 0.25×10^5 cell per insert onto the Matrigel Invasion Matrix (BD Biosciences, San Jose, CA, USA). Five per cent FCS was used as a chemoattractant. Invaded cells were stained with 0.005% Crystal Violet (Sigma) according to the manufacturer's instructions. Cells were counted per well and data were presented. Experiments were repeated in triplicate with each transfection condition performed in triplicate invasion chambers per experiment.

Determination of cell number

To determine the cell number in MCF-10A cells, transfected cells grown on the coverslips were fixed and stained with the nuclear stain, 4′,6-diamidino-2-phenylindole (DAPI) nuclei using methods described above. The slides were blinded to remove counting bias. Positively stained nuclei were counted, in two slides per transfection condition, and in total cells from three separate experiments.

Western blot analysis

Cells were lysed in 100 μl lysis buffer (5 mm HEPES, 137 mm NaCl, 1 mm $MgCl_2$, 1 mm $CaCl_2$, 10 mm NaF, 2 mm EDTA, 10 mm Na pyrophosphate, 1% Nonidet P-40, 10% glycerol and protease inhibitors (Roche)). Total protein concentration was measured using bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). In total, 50 μg cell lysate separated by SDS-PAGE using 10% SDS-PAGE gels were transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with 5% (w/v) milk protein in Tris-buffered saline containing 0.05% Tween-20. Blots were then incubated with primary antibodies as indicated, washed in Tris-buffered saline containing 0.1% Tween-20 and probed with primary antibodies at a dilution of 1:500 for LRH-1 (Abcam, Cambridge, MA, USA), 1:5000 for E-cadherin (BD Biosciences) and 1:10 000 for β-tubulin (Santa Cruz, Santa Cruz, CA, USA). Secondary IgG-conjugated mouse or rabbit antibodies, and Alexa fluor@ 700 (Invitrogen) were used at a dilution of 1:10 000 to visualise protein bands. Band intensities were quantified using the Odyssey infrared imaging system and Odyssey 3.0 Software (LI-COR Biosciences, Lincoln, NE, USA). Blots shown are representative of a minimum of three separate experiments.

Statistical analysis

All experiments were repeated at least three times. Data are presented as mean ± S.E.M. ANOVA or Student's independent *t*-test was calculated for experiments using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) and a P value of <0.05 was considered statistically significant.

Results

LRH-1 expression mammary epithelial cells lines

To ascertain *LRH-1* mRNA and protein expression in cell lines used in the following study, qPCR and western blot analysis were performed. As previously reported (Annicotte *et al*. 2005), the ER-negative MDA-MB-231 cells expressed lower levels of *LRH-1* mRNA compared to the ER-positive MCF-7 cell line ($P<0.05$; Supplementary Figure 1a, see section on supplementary data given at the end of this article). *LRH-1* mRNA was also detected, however, at lowest levels, in the non-tumourigenic mammary epithelial cell line, MCF-10A ($P<0.05$ when compared to expression in MCF-7 cells; Supplementary Figure 1a). However, western blot analysis demonstrated similar protein expression in all the three cell lines (Supplementary Figure 1a). Highest levels of LRH-1 was observed in the MCF-10A cell lines compared to MCF-7 and MDA-MB-231 cells ($P<0.05$; Supplementary Figure 1b), however, no difference in protein was observed between MCF-7 and MDA-MB-231 cells (Supplementary Figure 1b). Therefore, although ER-negative cell lines express less *LRH-1* mRNA than ER-positive cells, the protein expression is similar in all cells.

LRH-1 influences cell motility through actin remodelling in MCF-7 cells

To analyse the function of LRH-1 in breast cancer epithelial cells, endogenous LRH-1 expression was knocked down in the MCF-7 cell line using shRNA. Cells were transfected with control (shcontrol) or LRH-1-specific shRNAs (shLRH-1), and GFP expression from the tagged expression vectors indicated transfection efficiencies of ~70% (Fig. 1a). Twenty-four hours post transfection, shLRH-1 produced a 50% decrease in *LRH-1* mRNA ($P<0.001$; Fig. 1b) and immunoblots of whole cell lysate demonstrated knockdown of protein by 61% compared to control levels ($P<0.001$; Fig. 1c and d).

Cell migration is central to the process of EMT and plays a key role in cancer progression and metastasis. To characterise the effects of LRH-1 in breast cancer cell migration we used the wound-healing assay. LRH-1 knockdown significantly impaired the ability of MCF-7 cells to migrate into the created gap. Differences in wound healing were apparent from 6 h post wound initiation; closure of the gap was 21% in controls and 4% in LRH-1 knockdown cells (Fig. 1e). Following this trend, at 12 and 24 h, gap closure was ~10% at both time points in LRH-1 knockdown compared to 24 and 30% in control MCF-7 cells at 12 and 24 h ($P<0.01$ and $P<0.001$ respectively; Fig. 1e).

Actin re-arrangement into stress fibres and the development of protrusions from the outer cell surface mark the start of migration and are the hallmarks of EMT. To investigate the effect of LRH-1 on the development of lamellipodia and cytoskeletal rearrangements, phalloidin stain was used to visualise the actin cytoskeleton. Suppression of LRH-1 significantly reduced cell surface lamellipodia protrusions compared to controls at 24 h post transfection ($P<0.01$; Fig. 1f and g). The predominantly cortical actin arrangement in these cells was retained after LRH-1 suppression.

The initiating step in tumour metastasis is the dislodgement of cells from the primary tumour, and their subsequent invasion into adjacent tissues followed by metastasis. LRH-1 silencing caused a 20% decrease in cell invasion ($P<0.05$; Fig. 1h).

To extend the above observations, LRH-1 was over-expressed in MCF-7 cells and cell motility and actin structures were visualised as before. Transfection with an LRH-1 expression vector resulted in a 12-fold increase in *LRH-1* mRNA expression compared with vector-only-transfected cells ($P=0.005$; Fig. 2a). LRH-1 protein levels were increased twofold in LRH-1-transfected cells versus vector-only-transfected cells ($P<0.05$; Fig. 2b). Over-expression of LRH-1 accelerated migration into the created wound within 6 h post wound initiation (Fig. 2c and d). In over-expressing cells, 10, 29 and 31% gap closure was seen at the 6, 12 and 24 h time points while 2, 4 and 10% closure was observed at 6, 12 and 24 h in controls ($P<0.05$, $P<0.001$ and $P<0.001$ respectively; Fig. 2c–e). It is to be noted that control transfections with empty vectors (pcDNA or shRNA control) produced some variation in cell migratory patterns (Figs 1e and 2d). This is reflected in the differences in percentage gap closure in controls.

Appearance of filamentous actin protrusions were seen on the cell surface close to the wound edge in LRH-1 over-expressing cells compared to controls and quantitation of protrusion numbers indicated a twofold increase in LRH-1 over-expressing cells ($P=0.005$; Fig. 2e). Furthermore, MCF-7 cell invasiveness increased by 40% when LRH-1 was over-expressed ($P<0.05$; Fig. 2f).

LRH-1 influences cell motility through actin remodelling in MDA-231 cells

LRH-1 is known to stimulate expression of both aromatase (Bouchard *et al*. 2005) and is also an ERα target gene (Annicotte *et al*. 2005) in MCF-7 cells.

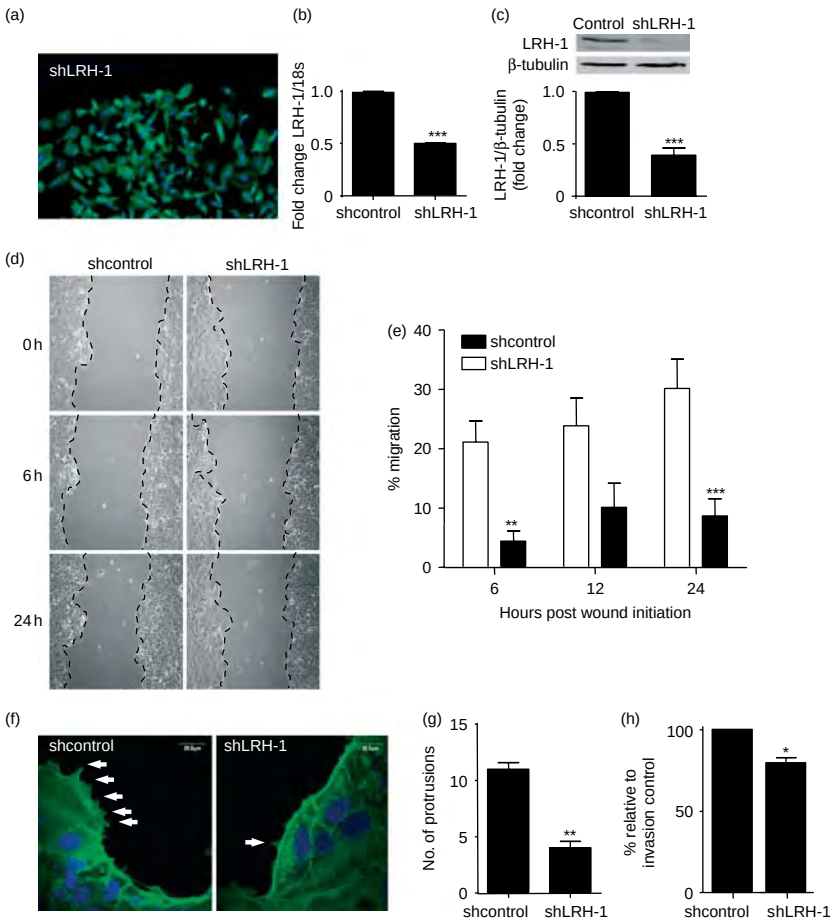

Figure 1 (a) Transfection efficiency was examined using GFP-tagged shRNA plasmid against the *LRH-1* gene (shLRH-1) in MCF-7 cells. (b) Real-time quantitation of *LRH-1* mRNA in the knockdown and control transfections demonstrated a 50% decrease in transcript levels. (c) Western blot analysis of whole-cell extracts collected 24 h post transfection with either shLRH-1 or control shRNA plasmids. Densitometric analysis revealed a reduction of LRH-1 expression of 60%, normalised to β-tubulin. (d) Wound-healing assay (×4 magnification) at 6, 24 and 48 h post wound initiation and (e) quantitation of migration following knockdown versus controls. (f) Cell morphology at the wound edge (×100 magnification), stained for actin fibres with phalloidin (green) and nucleus with ToPro (blue) show actin projections (white arrows) that were reduced at the wound edge in LRH-1 knockdown cells (g). (h) Invasion assays show reduction in invasiveness in LRH-1 knockdown cells. Data are presented as mean ± S.E.M., $n=3$ separate experiments, *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus controls.

To determine whether LRH-1-dependent cell migration occurs independently of oestrogen, we repeated these experiments using the ER-negative MDA-MB-231 cell line and obtained similar results (Supplementary Figures 2 and 3, see section on supplementary data given at the end of this article). LRH-1 knockdown cells showed 5 and 20% migration at 6 and 24 h time points respectively while scrambled shRNA control cells showed 18 and 47% migration (Supplementary Figure 2c and d). A 60% reduction in cell invasiveness was observed in LRH-1 knockdown cells compared to controls ($P<0.05$; Supplementary Figure 2e). Similarly, when LRH-1 was over-expressed, 30 and 70% migration was observed at 6 and 24 h compared to controls (15 and 43% at 6 and 24 h) ($P<0.05$ and $P<0.01$ respectively compared to controls) (Supplementary Figure 3b and c). As MDA-MB-231 is a highly invasive cell line, no further increase in cell invasion was measured in LRH-1 over-expressing cells (Supplementary Figure 3d).

LRH-1 enhances EGF-dependent migration and actin cytoskeleton changes in MCF-10A cells

To determine whether these effects of LRH-1 were confined to malignant breast cancer cells, experiments were repeated in the immortalised, non-tumourigenic mammary epithelial cell line, MCF-10A. LRH-1

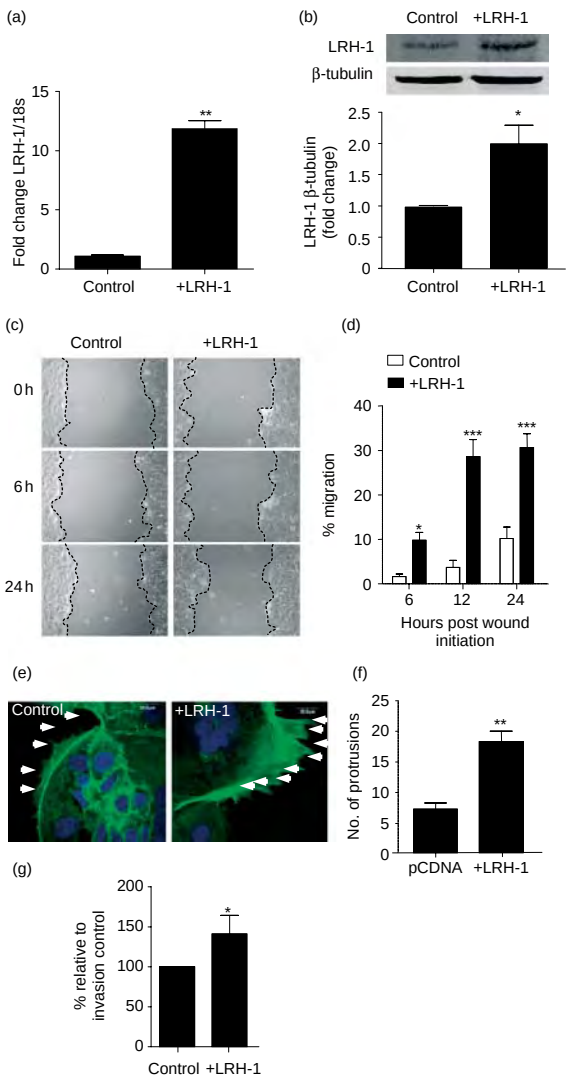

Figure 2 (a) Real-time quantitation of mRNA transcripts in LRH-1 over-expressing cells and control MCF-7 transfections demonstrated a 12-fold increase in transcript levels. (b) LRH-1 and β-tubulin immunoblots of lysates collected 24 h post transfection with either pCDNA–LRH-1 or pCDNA plasmids. Densitometric analysis revealed a 200% increase in protein. (c) Wound-healing assay (×4 magnification) at 6, 24 and 48 h post wound initiation and (d) quantitation of migration. (e) Cell morphology at the wound edge (×100 magnification), stained with phalloidin (green) and ToPro (blue) demonstrating actin projections (white arrows) at the wound edge were increased in LRH-1 over-expressing cells (f). (g) Cell invasion increased with LRH-1 increase in the cells. Data are presented as mean ± S.E.M., $n=3$ separate experiments, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus controls.

over-expressing cells had 62% cell migration compared to 20% in controls at 24 h post assay initiation ($P<0.05$; Fig. 3a and b). Over-expression of LRH-1 caused increased actin stress fibres in the cell architecture; membrane protrusions were observed and these rapidly formed connections with the neighbouring cells (Fig. 3c). Cells were also larger in size compared to control transfections ($P<0.001$) and were more elongated in structure (Fig. 3c). An increase in cell number was also observed following LRH-1 over-expression ($P=0.005$; Fig. 3e). As migration and cell proliferation were rapid following LRH-1 over-expression in MCF-10A cells, experiments were conducted at the 24 h point only.

The above-described experiments demonstrated that LRH-1 is directly involved in cell migration in both normal and malignant breast epithelial cells, and this effect is independent of ERα status.

LRH-1 over-expression causes post-translational cleavage of E-cadherin in conjunction with increased MMP9 expression

It is well established that alterations in E-cadherin function are correlated to increased malignancy and metastasis of epithelial cancer cells, and particularly related to EMT. The loss of E-cadherin function occurs through several mechanisms including transcriptional or methylation repression of the gene and post-translational truncation (Graff *et al*. 1995, Hajra *et al*. 1999). Therefore, we have studied the expression of E-cadherin using qPCR and western blot analysis in MCF-7 cells over-expressing LRH-1.

E-cadherin mRNA expression was significantly altered with changes in LRH-1 expression (Fig. 4a). With LRH-1 over-expression, a twofold decrease in *E-cadherin* mRNA was observed ($P<0.005$). Conversely, with the knockdown in LRH-1, a 0.5-fold increase in *E-cadherin* mRNA was noted ($P<0.05$). Western blot analysis indicated that cells with high LRH-1 protein expression (Fig. 4b and c) exhibited little or no change in the mature 120 kDa form of E-cadherin (E-cad^{120}) compared with control cells (Fig. 4b and e). In the LRH-1 over-expressing cells, however, there were higher levels of the truncated, inactive 97 kDa form of E-cadherin (E-cad^{97}) compared with control cells (Fig. 4b and d). Densitometric quantitation was used to determine the ratio E-cad^{97} to E-cad^{120} in control and LRH-1 over-expressing cells (Fig. 4f). With a twofold elevation in LRH-1 expression, the E-cad^{120} to E-cad^{97} ratio showed a twofold decrease; indicating a twofold increase in inactive E-cad^{97} levels (Fig. 4b, d and f). Since MMPs play important roles in cancer progression and are known to regulate E-cadherin cleavage, we examined MMP9 expression levels. Knockdown of LRH-1 expression in MCF-7 cells resulted in a 70% reduction in *MMP9* mRNA expression ($P<0.05$; Fig. 4g). Furthermore, in LRH-1

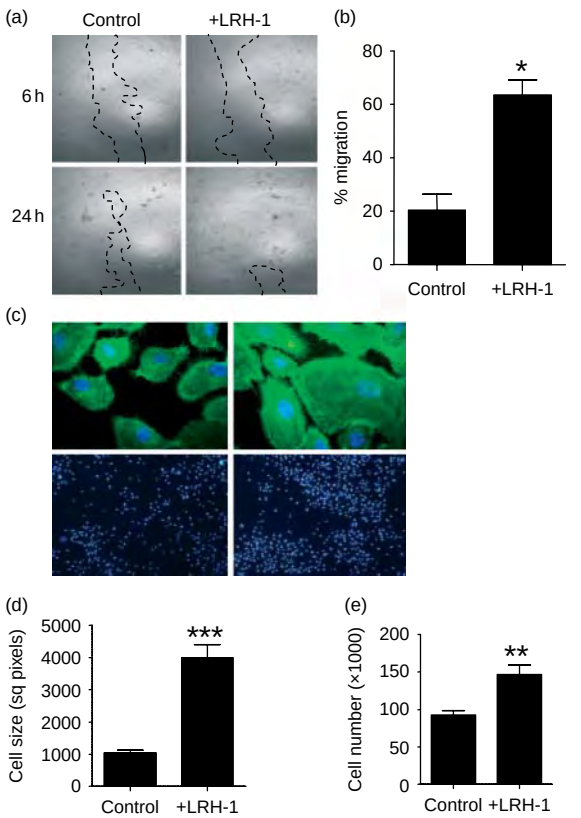

Figure 3 (a) Wound-healing phenotype (×4 magnification) and (b) quantitation of migration in MCF-10A cells following LRH-1 over-expression (+LRH-1) compared with cells transfected with plasmid vector only. (c–e) Photographs demonstrating differences in cell morphology and number: (c) cells photographed at ×40 magnification are stained with phalloidin (green) and DAPI (blue) at 24 h post wound initiation (top panel) and DAPI-stained cells photographed at ×10 magnification (bottom panel). Increases in cell size (d) and cell number (e) were observed with increased LRH-1 expression. Data are presented as mean ± S.E.M., $n=$ at least 3 separate experiments, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control.

over-expressing MCF-7 cells, a fourfold increase was observed in MMP9 expression compared with controls ($P<0.005$; Fig. 4h). These data suggest that the changes observed in E-cadherin may be mediated by the regulation of MMP9 expression by LRH-1.

Discussion

LRH-1 has well-established roles in embryonic development, cholesterol and bile acid metabolism and steroidogenesis. Recent studies have also highlighted its effects on cancer cell proliferation, particularly in tumours of the intestine and stomach (Botrugno *et al*. 2004, Schoonjans *et al*. 2005, Wang *et al*. 2008). In breast cancer, LRH-1 stimulates aromatase expression in adipose stromal cells, thereby increasing the availability of local oestrogen for ER-positive tumour growth (Clyne *et al*. 2002, Zhou *et al*. 2005). However, LRH-1 is also expressed in breast cancer epithelial cells where it could potentially have direct effects, independent of aromatase (Miki *et al*. 2006). In this study, using shRNA and over-expression strategies, we have shown that LRH-1 directly stimulates cell migration and invasion in both ER-positive and ER-negative breast cancer cell lines, as well as in the normal breast epithelial MCF-10A line. These effects are associated with actin remodelling, and post-translational inactivation of E-cadherin, suggestive of EMT.

LRH-1-induced motility and invasiveness was associated with decreased *E-cadherin* mRNA expression and cleavage of the membrane-bound E-cadherin protein (E-cad^{120}) to the inactive form (E-cad^{97}). In invasive breast and prostate cancer epithelial cell populations, the loss of cadherin-based adherens junctions is due to the cleavage, in the cytosolic domain, of E-cad^{120} to E-cad^{97} (Day *et al*. 1999, Vallorosi *et al*. 2000, Rashid *et al*. 2001). Facilitated by MMPs (Noe *et al*. 2001, Marambaud *et al*. 2002), this removes the β-catenin-binding domain from its cytoplasmic tail leaving a membrane-bound E-cad^{97} and a free cytoplasmic 35 kDa form (E-cad^{35}) still bound to β-catenin (Vallorosi *et al*. 2000).

The proteolytic processing of E-cadherin adherens junctions by MMPs plays a crucial cancer progression by allowing changes in cell adhesion, invasion, signalling and apoptosis (Vallorosi *et al*. 2000, Egeblad & Werb 2002, Marambaud *et al*. 2002, Lopez-Otin & Matrisian 2007). In LRH-1 over-expressing MCF-7 cells, we found an increase in *MMP9* mRNA expression. This LRH-1-dependent effect was validated in the knockdown experiments. Interestingly, evidence of LRH-1-dependent regulation of MMPs was demonstrated recently (Duggavathi *et al*. 2008). Using a granulosa cell-specific LRH-1 knockout mouse, a downregulation of MMPs (MMP2, MMP9 and MMP19) was observed (Duggavathi *et al*. 2008). The regulation of E-cadherin and MMP9 provides some evidence in support of LRH-1 promoting processes involved in EMT. Recently, breast cancer stem cells were shown to share EMT attributes (Mani *et al*. 2008, Blick *et al*. 2010), survival as individual cells outside the tumour and resistance of cells to the current therapies (reviewed in Polyak & Weinberg (2009)). Given the role of LRH-1 in ESCs and the implication that LRH-1 may support EMT in breast cancer epithelial cells, further analysis of LRH-1 in breast cancer stem cells is warranted.

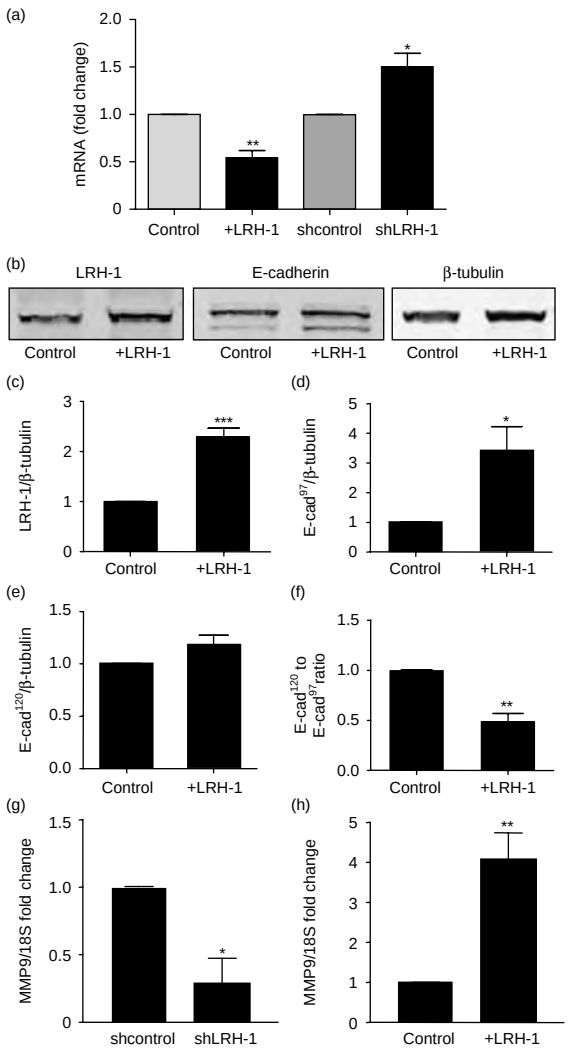

Figure 4 (a) E-cadherin mRNA expression MCF-7 cells with pCDNA (control), pCDNA–LRH-1 (+LRH-1), shcontrol and shLRH-1 plasmids. (b) LRH-1, E-cadherin and β-tubulin immunoblots of lysates collected 24 h post transfection with either pCDNA or pCDNA–LRH-1 plasmids. The densitometric analysis of immunoblots for (c) LRH-1, (d) E-cad^{97} (e) E-cad^{120} and (f) E-cad^{120} to E-cad^{97} ratio in control and +LRH-1. The E-cad^{120} to E-cad^{97} ratio in +LRH-1 cells reflects an increase in the inactive, truncated E-cad^{97} form. MMP9 mRNA expression was analysed in (g) shLRH-1 and (h) +LRH-1 cells. Data are presented as mean ± S.E.M., n= at least 3 separate experiments, *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus controls.

Our data suggest that LRH-1 has both direct stimulatory effects on breast cancer cell motility and invasion, in addition to indirect proliferative effects mediated by activation of aromatase expression (Clyne *et al*. 2002, Zhou *et al*. 2005). It is therefore an attractive therapeutic target. As an orphan receptor, however, no classical ligands for LRH-1 have been identified. Recently, the crystal structure of the LRH-ligand-binding domain revealed the presence of bacterial phospholipids derived from the *Escherichia coli* expression system used to produce the receptor (Krylova *et al*. 2005, Ortlund *et al*. 2005, Wang *et al*. 2005*a*). At present it is unclear whether mammalian phospholipids bind to LRH-1 endogenously, although they can readily be exchanged for bacterial lipids bound to the LRH-1 homologue SF-1, *in vitro* (Sablin *et al*. 2009). Irrespective of the existence or otherwise of an endogenous ligand, the presence of a conserved large hydrophobic pocket with the LRH-1 LBD should allow the identification of synthetic ligands that could fit into this space and potentially modulate LRH-1 activity. Indeed, compounds that bind to LRH-1 and displace bound phospholipids have been described, however, these functioned as agonists rather than antagonists (Whitby *et al*. 2006); to date, no small molecule LRH-1 antagonists (or inverse agonists) have been identified.

It is thought that LRH-1 activity is regulated primarily by the recruitment of co-activator (Xu *et al*. 2004) or co-repressor (Sablin *et al*. 2008) protein partners. Recently, we have described a series of short peptides, identified by phage display, that bind to the LRH-1 LBD and inhibit recruitment of co-activators (Safi *et al*. 2005). These peptides acted as potent antagonists, completely inhibiting LRH-1 transcriptional activity and its ability to stimulate aromatase promoter activity. Although peptides are not ideal drug candidates, small drug-like molecules that inhibit the interactions between other nuclear receptors and their co-activators have recently been discovered (Arnold *et al*. 2005). Taking a similar approach may identify candidate inhibitors of LRH-1:co-activator interactions that block both its direct proliferative and indirect oestrogenic effects in breast cancer cells.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-10-0179.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Health and Medical Research Council of Australia (C D Clyne), the Victorian Cancer Agency (A L Chand), the Cancer Council Victoria (C D Clyne), the United States Department of Defense (BC084667) (E W Thompson) and by the Victorian Government's Operational Infrastructure Support Program.

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11 The Continuum of Epithelial Mesenchymal Transition – Implication of Hybrid States for Migration and Survival in Development and Cancer

Lilian Soon, Anthony Tachtsidis, Sandra Fok, Elizabeth D. Williams, Donald F. Newgreen, and Erik W. Thompson

EMT AND CELL MIGRATION – EMBRYONIC NECESSITIES CO-OPTED BY INVASIVE CANCER

The concept of the epithelial–mesenchymal transition (EMT) originated from studies of events in development, particularly those preceding the onset of cell migration [1]. These were initially brought together and popularized by the efforts of the late Elizabeth Hay and colleagues [2, 3]. One of the most intensively studied examples of EMT and cell migration is the generation of migratory neural crest mesenchyme from the neurectodermal epithelium [4] (Figure 11.1). These cells go on to form the autonomic and sensory nervous systems. Even prior to this, the emergence of the primary mesenchyme from the epithelial epiblast during gastrulation (the first EMT) results in the formation of highly motile cells that are critical to the development of the body plan [1] (Figure 11.2A). Further EMTs occur in other epithelia after the neural crest EMT to generate the cells that form muscle, bone, and connective tissues (Figure 11.2B). Such cellular plasticity is fundamental to embryological development and is regulated largely at the transcriptional level. Various transcriptional repressors of E-cadherin (and other cadherins), such as Snail (Snail 1), Slug (Snail 2), Twist, Zeb1 (δEF1), Zeb2 (SIP), and E47/E12, regulate EMT in developmental system (reviewed in [5]).

Commitment to lineage differentiation in normal cells is more pliant than first thought, and cellular transition is emerging as a major mechanism of adult tissue homeostasis [6].

The descriptive similarity of developmental EMT and cell migration to local tissue invasion from a carcinoma is striking, suggesting that the latter is a pathological EMT [7–9]. The similarities have been extended down to the level of molecular expression and gene regulation, although, it must be admitted, mostly using in vitro models employing cancer-derived cell lines [10]. Direct evidence in human or animal cancers has been elusive, fueling a controversy as to the reality of EMT in cancer invasion [11]. However, more recent in vivo approaches employing markers whose expression is driven by regulatory elements of EMT-associated genes have provided stronger "guilt by association" evidence of EMT occurring at sites of invasion in real tissues (reviewed in [12, 13]). This has been supported functionally; when cell suicide was linked to EMT gene expression it resulted in reduced metastasis in animal models [12].

Evidence of EMT-like traits in cancer cell lines has been endorsed by recent genome-wide Affymetrix profiling studies of large collections of human breast cancer cell lines comprising thirty-four [14] and fifty-one [15] cell lines (reviewed in [16]). At the same time, the concept has been raised of cancer stem cells (rather than the bulk of the cancer cells) as a disproportionately important factor in cancer growth and dissemination. This is consistent with early reports of migrating cancer stem cell attributes in cells exhibiting EMT properties at the invasive front of gastric cancers [17]. The stem cell concept has become confluent with that of cancer EMT, with commonality between the EMT and the breast cancer stem cell (BCSC) transcriptional makeup derived by Serial Analysis of Gene Expression [18]. Indeed, BCSC-like attributes can be seen in human mammary epithelial cells after EMT induction [19]. From an etiological point of view, processes that occur in a small fraction of cancer cells may play a key role in the phenotype of the overall disease. Metastatic disease may be established from single cells recurrently, and EMT may play a key role in metastatic dissemination of these cells, with only a minute fraction of the carcinoma cells in the primary site exhibiting EMT markers. It is for this reason that BCSC biology draws significant attention in recent research [20].

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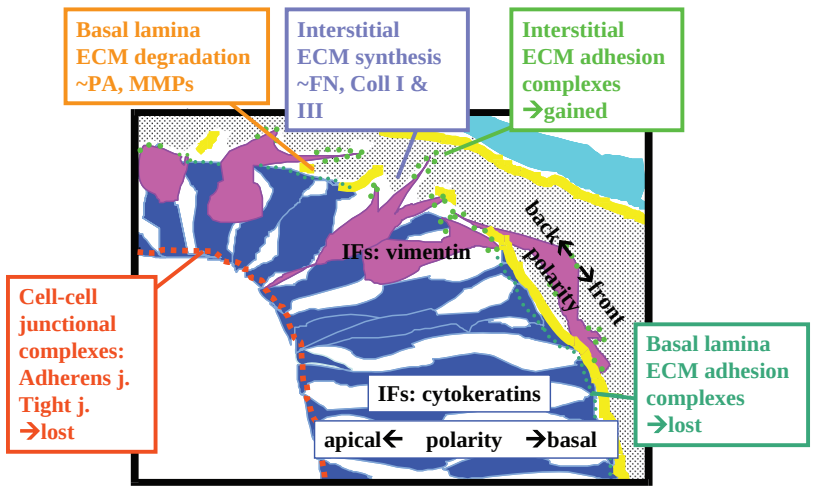

Figure 11.1. Neural crest EMT. Characteristic changes in EMT shown in a diagrammatic transverse section of the neural crest example. Neural epithelial cells (blue) convert to mesenchymal neural crest cells (pink) with changes in polarity and intermediate filaments (IF). Cell–cell adhesions are reduced and cell–ECM adhesions are modulated. In addition, proteases such as plasminogen activator (PA) and MMPs are upregulated, and ECM is altered by both degradation and synthesis.

THE EMT SPECTRUM – EXTREME TO PARTIAL

Extreme examples of any biological phenomenon lend themselves to study; this is true of the study of EMT and its immediate aftermath, cell migration/invasion. From these studies, the defining features of EMT and migration (see Figure 11.1) include:

1. Downregulation of cell–cell adhesion, particularly due to loss of classic cadherin-mediated junctions. This enables the new mesenchymal cells to separate from former epithelial neighbors.
2. Reorganization of the cytoskeleton (exemplified by F-actin changes) leading to loss of apicobasal polarity typical of epithelial cells and gain of locomotor (front–back) polarity. This leads to degeneration of the highly structured epithelial pattern and, potentially, to cell migration away from the site.
3. Altered expression of genes for molecules of the extracellular matrix (ECM), and for the cell surface adhesion molecules (particularly the integrins) that mediate cell–ECM adhesion. This allows cells to rearrange with respect to basal lamina versus interstitial ECM, and potentially to gain traction on the latter.
4. Upregulation of genes encoding extracellular proteases, such as plasminogen activators and diverse matrix metalloproteases (MMPs), potentially allows cells to remove cell–cell adhesive molecules, enables cells to pass through basal lamina ECM, and facilitates penetration of dense interstitial ECM.

In examples such as gastrulation or neural crest migration in mammalian or avian embryos, and in certain cancer cells in vitro, these features are carried through to a high degree, and the mesenchymal cells are regarded as behaving individualistically. However, when even extreme EMT and migration events, such as avian neural crest cell migration, have been studied dynamically by time-lapse imaging in real tissues, the cells are seen to have strong social interactions that govern their behavior. Neural crest cells, when migrating, are almost always in contact with other neural crest cells and form "head-to-tail" chains. The contacts between individual cells are measured in minutes and neighbor exchange is frequent. When deprived of neighbors, however, neural crest cells cease directionally persistent locomotion, and hence show minimal real migration or invasion [21, 22].

Much less extreme situations also exist, in which some EMT features occur but not others, or the level of the feature is less than extreme. A developmental example of this is gastrulation in frogs. This process is clearly homologous to gastrulation in mammals and birds, but instead of the gastrulating cells migrating as an apparently disorderly mob of individualistic cells, the cells spread as a sheet with the advancing edge cells showing front–back polarity and motile specializations, but maintaining intimate epithelial-like cell–cell adhesions to the cells behind the front. This kind of sheet-like spreading resembles wound healing in epithelia. Many other examples of movement of groups of cells, or collective cell movement, are known [23]. These include the extension of cells as coherent cords in angiogenesis; tubulogenesis in nephric, mammary, and lung morphogenesis; the movement of essentially a ball of border cells in *Drosophila*; movement of cell clusters in early vertebrate heart morphogenesis; and the behavior of many cancer cells in various in vitro assays (as summarized later). The terminology applied to these diverse cases in not yet agreed on, but they clearly share many features of EMT; such "epithelial" cells have been termed "relaxed," "activated," "hybrid," "metaplastic," or "metastable" with a "partial EMT."

MET – BACK TO THE FUTURE

Developmental EMT and cell migration is often followed by a later phase whereby the migrating cells cease migrating and aggregate. When cells in some lineages are followed through time, cycles of EMT and mesenchymal–epithelial transition (MET) have been revealed. The classic developmental example of this is the epithelial epiblast lineage that generates the

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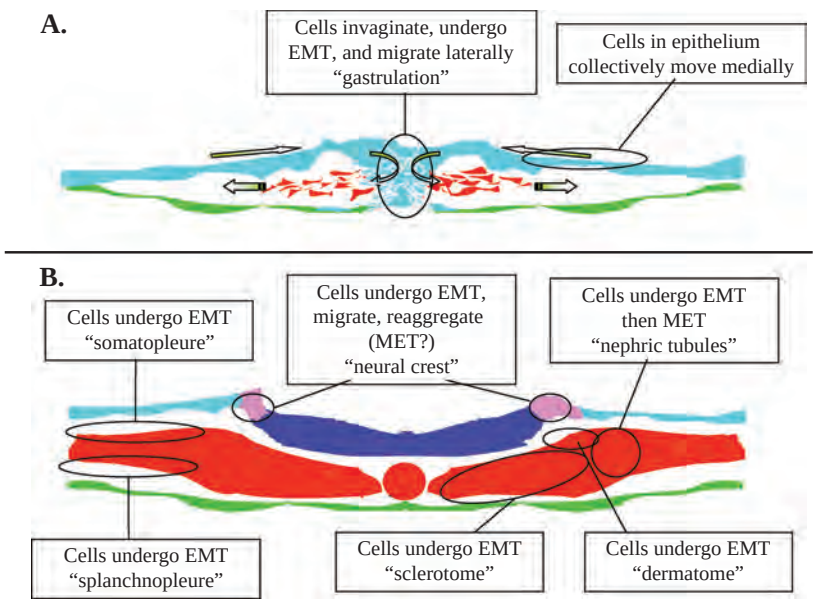

Figure 11.2. Developmental EMTs. **A.** The first EMT in metazoans is gastrulation, shown in transverse section. The upper epiblast epithelium cells collectively move medially, then invaginate and undergo EMT at the midline. The resulting mesenchymal cells (red) form a new and apparently disorderly middle layer, with the cells moving individualistically laterally. **B.** After gastrulation, the mesenchyme cells condense to form new epithelia (mesoderm), which undergo complex patterns of further EMTs and METs. Several stages of development are represented in this one diagram.

primary mesenchyme (via an EMT) during gastrulation (Figure 11.2A). This primary mesenchyme then forms epithelial layers termed the *segmental plate* of presomitic,,intermediate, and lateral plate mesoderms (via a MET). The segmental plate mesoderm, after reorganizing into rosette-shaped epithelial somites, forms the mesenchymal sclerotome (another EMT). The lateral plate mesoderm (termed *somatopleure* and *splanchnopleure*) also disperses to mesenchyme (EMT), whereas the intermediate mesoderm disperses (an EMT) but goes on to later reepithelialize to form nephric tubules (MET) (Figure 11.2B). The notion that cells after EMT might not be irreversibly bound to this state, and indeed might revert via MET, has been embraced in the field of metastasis.

CANCER AND THE EMT CONCEPT

Developments in gene and molecular expression provide depth to the enormous increase in studies documenting EMT and associated functional changes in culture systems (for recent reviews, see [24–28]) and the increasing number of studies attributing prognostic significance to EMT indicators in the primary tumor. These include specific studies of EMT markers, such as vimentin, which is typically expressed by cells of mesenchymal origin, although some controversy still exists in respect to its prognostic significance in breast cancer [29]. Recently, a collection of EMT-like markers, including vimentin, were linked to the more aggressive "basal" subtype of breast cancer [30]. Other studies have shown prognostic significance to EMT drivers, such as the E-cadherin repressors mentioned above. These include Snail (Snail 1), Slug (Snail 2), Zeb1 (δEF1), and Twist [31–40].

Such studies are not limited to breast cancer. Baumgart et al. [41] investigated the prognostic significance of several markers known to be linked with EMT in clinical samples of varying stage and grade of bladder tumors. It was found that a loss of E-cadherin, accompanied by a reduction and relocalization to the cytoplasm of β-catenin and plakoglobin, were associated with late tumor stage and grade. This study also determined an upregulation of N-cadherin and vimentin expression in some of the tumor samples examined. In addition, several more specific studies investigating E-cadherin expression in particular, along with a range of physically, functionally, and mechanistically associated molecules (such as moesin, zyxin, α-catenin, p53, RB, and INK4A), were performed [42–47]. Several of these studies investigated E-cadherin expression in clinical samples with respect to its possible prognostic value; however, these studies provided variable results. Thus, despite the large amount of data implicating some of the EMT-related processes in tumor progression, clinical data remain somewhat problematic and the definitive EMT marker has not yet emerged. Many laboratories, including our own, are pursuing markers that may better distinguish breast and other cancers that have a propensity for EMT.

Several studies link the process of EMT, and presumably also plasticity around the EMT axis, to the actual metastatic process. Extraordinary demands are placed on epithelial-derived carcinoma cells to successfully metastasize, including separation from the epithelial collective, degradation of the surrounding matrix, migration and invasion through the basement membrane, intravasation and survival in the circulation, extravasation at a secondary site, survival as micrometastases, and finally growth into overt metastases [48]. To complete these complex steps, cancer cells exhibit both mesenchymal- and epithelial-like properties at different times, or even at the same time [3, 26, 28]. EMT regulators do alter the cell cycle machinery of cells, and through these means may allow the prolonged survival of residual cancer cells [49]. Indeed, the EMT regulator Snail 1 was implicated in the emergence

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of residual disease into local recurrence after oncogene silencing [50].

Mesenchymal derivatives of carcinoma cells show a number of attributes that would favor metastasis, such as separation from the collective as individual cells, increased migratory and invasive potential, increased survival in suspension, and resistance to apoptosis in response to chemotherapy. Sustained expression of mesenchymal traits would assist in extravasation at the metastatic site, and possibly also survival at that site. Some anecdotal evidence has accrued on the likelihood that circulating tumor cells (CTCs) and micrometastases show EMT characteristics. It has long been recognized that CTCs show reduced expression of specific cytokeratins [15], which are regarded as epithelial hallmarks, and it was recently shown that cell lines derived from breast cancer micrometastases stably express the mesenchymal marker vimentin [51].

In addition to translocation during metastasis, EMT biology has grown to encompass resistance to anoikis [52], enhanced survival [49], genomic instability [53], and resistance to chemotherapies [54], and thus represents a potentially comprehensive target in cancer biology. By commandeering developmental EMT pathways, sessile epithelial carcinoma cells are transformed into cells with migratory and invasive capability, metastatic potential, and a resistance to anoikis and chemotherapy [55].

CANCER AND THE MET CONCEPT

The concept of the MET has also found application the cancer field, in which the establishment of metastases has been suggested to involve a MET-like reversion of the EMT that enabled the initial escape from the primary tumor site. This is consistent with the well-established similarity of primary and secondary tumors [56–60]. In one of the clearest examples, metastatic colorectal carcinoma, metastases distant from the primary site may adopt a morphogenesis and differentiation pattern closely resembling colonic epithelium [17]. Studies in our own laboratory showed that variants of the metastatic T24/TSU-Pr1 bladder carcinoma line selected for metastatic potential express more epithelial markers (cadherins and keratins) than their less metastatic counterparts [59, 61]. Furthermore, in prostate cancer metastasis to the liver, upregulation of the epithelial marker E-cadherin was observed on cancer cells; this increase in E-cadherin could be modeled by the co-culture of prostate cancer cells with hepatocytes [62]. In addition, fluorescent markers driven by epithelial and mesenchymal-related FGF receptor isoforms in prostate cancer cells have highlighted the epithelial–mesenchymal plasticity both in the primary site and in lung metastases. This is in accord with the notion that the ability to convert between the epithelial and mesenchymal states is most effective in allowing cells to both leave the primary tumor and to establish a distant metastasis (Figure 11.3). Indeed, careful analysis of EMT-derived populations suggests that the "hybrid" or metastable phenotype is more prevalent than pure mesenchymal derivatives. We have found coexpression of both epithelial and mesenchymal markers in the PMC42LA human breast cancer system of epidermal growth factor (EGF)-induced EMT (discussed later). These cells exhibit coexpression of vimentin and epithelial cytokeratins [63], as well as EpCAM (unpublished).

This hybrid phenotype reflecting epithelial–mesenchymal plasticity has become well-recognized recently in cancer systems [25], and has been referred to as a *metastable phenotype* [64] or activated epithelium [65]. Others have also reported this hybrid state [66]; this could partly explain the difficulty of observing EMT in clinical samples [11]. Acquisition of mesenchymal characteristics may be transitory, may occur on a background of epithelial gene expression, and may be reversed during metastasis.

MOLECULES OF EMT AND MET

The most reliable piece of evidence that establishes a continuum between complete EMTs and the partial or less obvious forms is the expression of genes and molecular functions in common between development and cancer. EMT is a culmination of changes affecting adhesion components and their associated signaling pathways in a manner that promotes the migration of cells in settings such as gastrulation during development or metastasis in cancer [67]. An example would be the downregulation of E-cadherin, which may occur as a result of receptor tyrosine kinase (RTK) activation upregulating MAPK or Wnt signaling pathway activity, which in turn blocks the ability of GSK3β to repress MAPK activity [26, 28]. Both pathways allow an increase in Snail 1 and Snail 2 activity, blocking E-cadherin transcription [68–70]. Other changes as part of EMT include RTK activity in reducing the assembly and stability of adherens-junction components, as well as the breakdown of tight junctions through transforming growth factor β (TGFβ)-induced depolymerization of cytoskeletal elements [28]. A summary of the signaling components common to cancer and developmental EMT is shown in Table 11.1.

THE SHOP FLOOR OF EMT–CELL ADHESION AND CYTOSKELETAL MOLECULES

The archetypical epithelial cadherin (E-cadherin) effects homotypic adhesion between most adult epithelia, through the adherens junctions [71]. Regulation of the various types of cadherin and their expression

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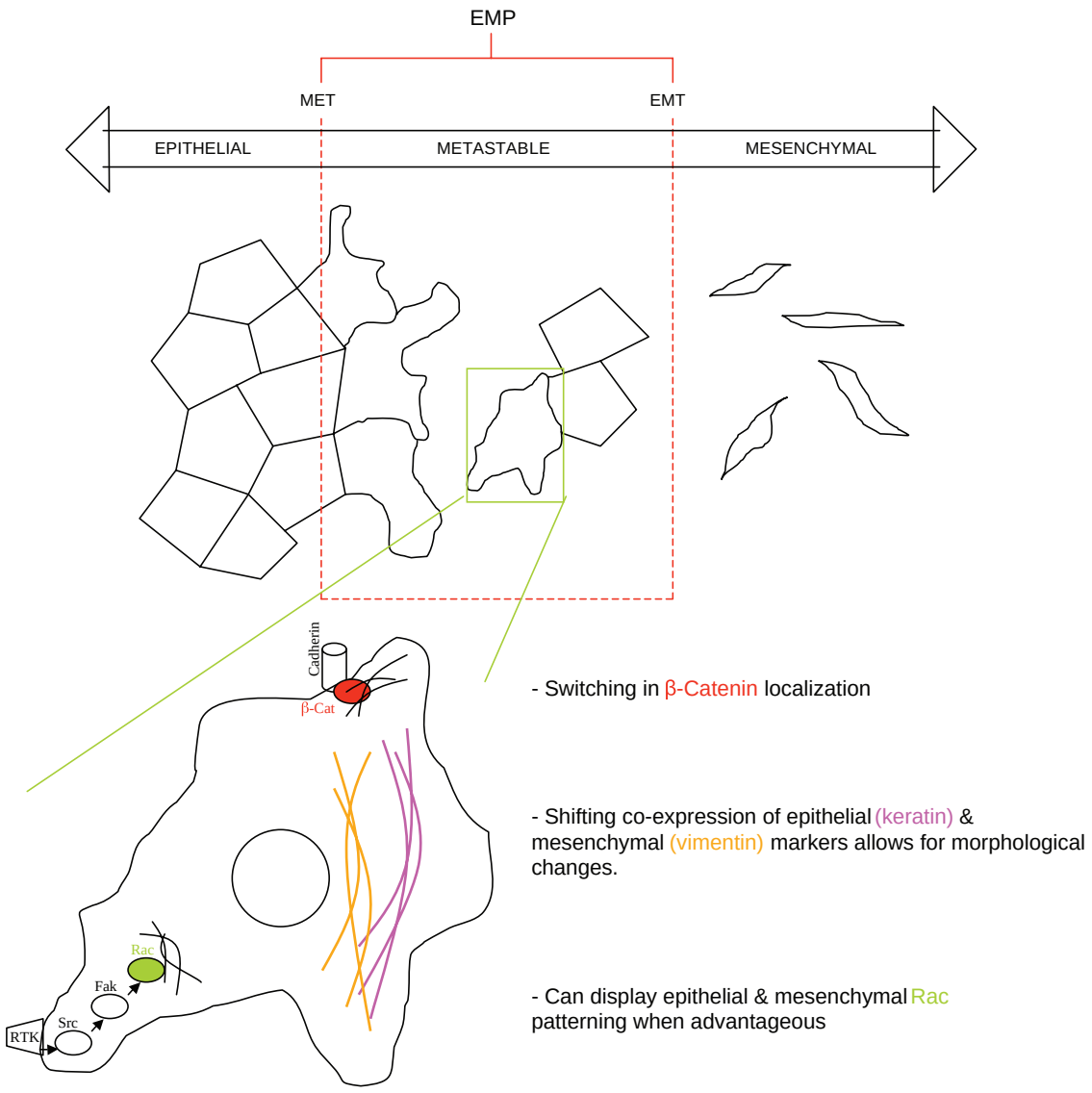

Figure 11.3. The hybrid/metastable phenotype. The "metastable" cell, as indicated in the lower panel, may embrace features that allow the cell to readily switch between epithelial and mesenchymal phenotype. This subpopulation of cells may be key in allowing the various steps of metastasis to be accomplished. Three aspects that the metastable cell could give it a selective advantage include (i) shifts in Rac patterning; Rac is involved in cytoskeletal and focal adhesion rearrangement; (ii) β-catenin localisation; β-catenin is important in strengthening cell–cell adhesion by stabilizing the intracellular tail of cadherins to cytoskeletal elements; (iii) coexpression of epithelial and mesenchymal markers, such as vimentin, a well-known mesenchymal intracellular filament, and epithelial keratins. Readily shifting the scale of these (among others) would allow for a more epithelial or mesenchymal phenotype when required. EMP: epithelial-mesenchymal-plasticity, MET: mesenchymal-epithelial transition, EMT: epithelial-mesenchymal transition.

levels profoundly influences cell properties. For example, several cadherins are expressed during kidney organogenesis, including E-cadherin (cadherin-1), N-cadherin (cadherin-2), R-cadherin (cadherin-4), and K-cadherin (cadherin-6). Interestingly, E-cadherin is first detected after induction of the metanephric mesenchyme, and blocking antibodies against E-cadherin do not affect MET [72]. These findings suggest that an alternative cadherin (or other type of adhesive molecule) is responsible for the induction of the epithelial phenotype during kidney organogenesis. Notably, both K-cadherin$^{-/-}$ and R-cadherin$^{-/-}$ mice display defects in MET [73]. N-cadherin can stimulate the FGF receptor, which in turn upregulates MMP activity [74]. This has a number of effects, including cleavage of the extracellular domain of other cadherin molecules. For example, MMP-3 or MMP-9 can cleave the extracellular domain of E-cadherin [75]. Cleaved fragments

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TABLE 11.1. Molecular Parallels between Cancer and Normal EMTs*

Molecule	Cancer-related function	Normal function
Adhesion Molecules		
Cadherins	E-cadherin suppression causes EMT	Cell–cell adhesion
Integrins	Disruption of cell–matrix adhesion and signal	Cell–matrix adhesion
Extracellular Factors (and Receptors)		
IGF	Can activate Ras/Raf, PBK/Akt via RTK	Growth
HGF	Can activate PBK/Akt via RTK	Growth, motility, morphogenesis
EGF	Can activate Ras/Raf, NF-$\kappa\beta$ activity via RTK	Cell division
FGF	Can activate Src/Rac, Ras/Raf, PBK/Akt via RTK	Growth, morphogenesis, division, tissue repair, embryonic development
MMP	Break down and remodel tumor microenvironment to allow for growth and invasion	ECM breakdown, tissue remodeling
BMP	Can activate Smad signaling pathways	Cell fate, induces neural crest and other EMTs, stimulates ectopic bone growth
Jagged	Binds to and activates Notch	See "Notch"
Wnt	Can block GSK3β activity	Cell fate, patterning during embryogenesis
TGFβ	Interacts with the bulk of proteins listed below	Proliferation, differentiation, has immune function
Signaling Proteins		
Smad	Can activate LEF1	Transcriptional regulators
Rho GTPase	Can activate PβK, ROCK (involved in actin stress fiber formation)	Small G protein, regulates intracellular actin dynamics
Ras/Raf	Activates MEK-ERK pathway, causes transcription of EMT promoting genes; activates PβK, MAPK	Regulating adherens junctions, focal adhesions, myosin phosphorylation, actin stress fiber formation
Src/Rac/ROS	Activate Snail	Embryonic development, cell growth
PI3K	Interact with Ras, Akt, ILK	Embryo implantation in the uterus
Notch	Activate H/Espl transcriptional regulators (e.g. Heyl/Hey2/Hesl/Hes5)	Fate determination during development
GSK3β	Block MAPK and NF-Kβ	Metabolism, neuronal cell development, body pattern formation
NF-$\kappa\beta$	Promote Snail	Important in regulating immune response to infection
MAPK	Promote Snail/Slug, Jun/Fos, Ets	Gene expression, mitosis, differentiation, apoptosis/survival
ILK	Activate Snail	Important for integrin function during development
Nuclear Regulators		
Snail/Slug	Suppress cadherin transcription including E-and N-cadherin. Protein binds E-cadherin gene regulatory E-box.	Promotes EMT in multiple systems. – Associated with function of signal protein, which governs this nuclear regulator
Id	Inhibit E2A	Differentiation inhibitor. Growth, angiogenesis and apoptosis regulation.
Twist	Inhibit HOXD10 and RHOC, increasing motility. Suppress E-cadherin transcription (see Snail).	Cell identity. Promotes EMT. Mesoderm differentiation and neural crest cell migration
H/Espl (Heyl/ Hey2/Hesl/ Hes5)	TGF-β induced H/Espl = positive regulation of Snail, repress VE-cadherin; targets of H/Espl in EMT still need further identification	Embryonic segmentation, cardiac development.
δEF1/ZEB1	Suppress cadherin transcription (see Snail). Target of mIR200 suppression.	Promote EMT in multiple systems. Segmental patterning and morphogenesis of many systems.
SIP1/ZEB2	Suppress E-cadherin transcription	as δEF1/ZEB1.
Fos	Suppress E-cadherin transcription	Regulation of angiogenesis, cell proliferation and apoptosis
LEF1	Promote vimentin, MMP	Mesenchymal gene program (co-trancription factor with β-catenin.
E2A	Repress E-cadherin	Control angiogenesis, cell proliferation and apoptosis in many cell systems.

* Classes of molecules implicated in both development and cancer EMTs are summarized. Much of the described signaling is associated with modulation of cell–cell adhesion components that allow for EMT. However, their ability to regulate other aspects of the cell (e.g. intracellular framework, ECM adhesion, activation/inhibition of other EMT molecules) can occur in concert with one another as well as with the functions listed above. The list of molecules is not complete, nor are the described signaling pathways with which they interact exclusive. There is significant crosstalk among many of the pathways described above and the molecules associated with them, as well as with a plethora of others not described here. Additionally, cellular context and the nature of signaling pathway activation/inhibition and expression will determine which one of the many functions a single molecule may have.

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compete with intact cadherins for binding to adjacent cell cadherins, reducing cell–cell adhesion and promoting EMT. In neurogenesis, this is important in rearranging cells to form developing structures. In cancer, this may be a mechanism to promote metastasis, facilitating tumor cell migration away from the tumor mass. Catenins are key molecules involved in cadherin function. They modulate cadherin clustering and strength of the cadherin–actin cytoskeletal connection. In addition, $p120^{ctn}$ regulates cadherin turnover by promoting cadherin stability [76].

Although the majority of studies investigating the role of cadherins have been EMT-directed, it is likely that cadherins are involved in the development of the cohesive tumor mass at the secondary site. Cellular and tissue context plays an important role in determining the action of any given cadherin. N-cadherin has been observed to cause vigorous cell adhesion in multiple cell types, including cardiac muscle [77]. Conversely, it has been found to promote cell motility and scattering in other cell types, such as endothelial cells in blood vessels undergoing angiogenesis [78]. N-cadherin generates migratory signals through distinct pathways [79].

EMT IMPLICATIONS FOR CELL LOCOMOTION

A typical epithelium is a sheet of cells, polarized at apical and basal surfaces [10]. Mesenchymal cells generally exhibit neither regimented structure nor tight intercellular adhesion, but do show some leading-edge polarity when motile. Mesenchymal migration is mechanistically different from epithelial movement, in which cells move as a sheet en bloc. Mesenchymal migration is considerably more dynamic and, at first sight, unruly. During EMT, otherwise sessile collectives of epithelial cells downregulate cell junctional machinery (adherens junctions, tight junctions, and desmosomes) and gain motility.

MESENCHYMAL AND AMOEBOID MIGRATION AND INTERCONVERTIBILITY BETWEEN MIGRATION STATES

Individual cancer cell movements have been described as either mesenchymal or amoeboid [80]. The transition between the different states is thought to occur from epithelial to mesenchymal to amoeboid [81]. These states are, to some extent, interconvertible, depending on the molecular expression of genes and the microenvironment of the cell. In any given cancer or cancer-derived cell line, there is some degree of heterogeneity, a characteristic that is beginning to be recognized and explored. When investigated at the single-cell level, it is clear that cell lines are composed of mesenchymal-like and amoeboid-like cell types [82]. It remains unclear what the relative contribution of each subpopulation is to cancer development and what the resulting implications are for cancer treatment.

During mesenchymal cell migration, a cell protrusion extends anteriorly and draws collagen fibers posteriorly, a process that couples tightly with adhesion and cell contraction in the movement cycle. Mesenchymal migration is dependent on Rac activity and protease secretion but not on ROCK-mediated actomyosin contraction. The activation of Rac by NEDD9 and DOCK3, a GEF for Rac, promotes mesenchymal-type migration. In concert with its downstream effector WAVE2, Rac also suppresses amoeboid movement by reducing actomyosin contractility [83]. In the presence of protease inhibitors, mesenchymal cells can convert to amoeboid migration and continue to invade into the matrix.

Amoeboid migration, on the other hand, requires actomyosin contractility regulated by Rho and ROCK, but proteases are dispensable owing to the ability of the cells to move through the matrix sieve by cell contraction and hydrogel propulsion [84]. Amoeboid cells suppress mesenchymal movement through Rho-kinase-mediated activation of ARH-GAP22, a Rac GAP that causes Rac inactivation [83]. It is possible to convert amoeboid cells to a mesenchymal migration phenotype through expression of an activated form of cdc42 [85]. These cells now assume a more elongated morphology and require proteinases for invasion.

Although cdc42 is thought to be necessary for both amoeboid and mesenchymal migration, its downstream effectors have more specific roles. For example, DOC10, a GEF for cdc42, is necessary for amoeboid migration. Abrogation of DOC10 results in conversion from amoeboid to mesenchymal migration with reduced myosin light chain phosphorylation and increased Rac activation [85]. Its effectors NWASP and PAK also serve to maintain the amoeboid phenotype. However, blocking cdc42 suppressed the mesenchymal phenotype suggesting that although cdc42 is important for both amoeboid and mesenchymal migration, different effectors may functionally distinguish the two migration modes.

Amoeboid movement is represented by either a blebbing, contraction-mediated mode or a protrusion-centred movement found in leukocytes. Blebs occurring during migration are small, hemispherical membrane protrusions present at the cell periphery (Figure 11.4). Blebbing has been described to occur in cortical actin regions that are susceptible to breakages, allowing the outflow of cytoplasm and the extension of plasma membrane (PM) to form blebs [84, 86]. These structures are roughly 2 μm in diameter and are transient, existing for about one minute before retracting into the PM. In stimulated cells, extracelluar activation initially causes the destabilization or depolymerization of actin at a region of the PM and hydrostatic pressure, then drives cellular cytoplasm and the PM to form a bleb. The expansion of

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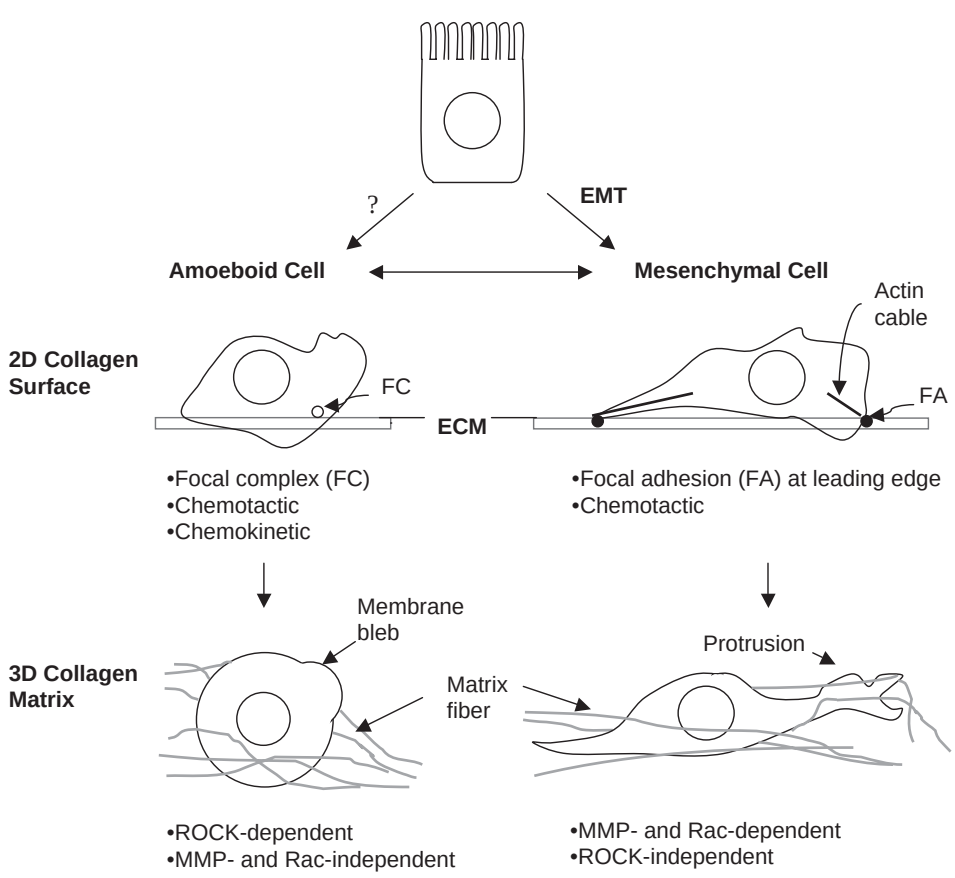

Figure 11.4. Modes of migration. Cell transformations following EMT produce amoeboid- and mesenchymal-like tumor cells that move in characteristic manners on two-dimensional and within three-dimensional environments. Amoeboid cells express transient and weak focal complexes that lie within the lamellipodium, whereas mesenchymal-like cells produce focal adhesions that make strong contact with the substratum. Amoeboid cells are efficient at both chemotaxis and chemokinesis, but mesenchymal cells move efficiently under gradient conditions only (as tested in Boyden chambers). In time-lapse imaging, amoeboid cells tend to move in random directions owing to a propensity to produce a new lamellipodium in different directions. Mesenchymal cells, on the other hand, are more persistent in moving in a single direction according to the polarity of the cell. When seeded in three-dimensional matrices and in tumors, amoeboid cells move through matrix pores by contraction of the cortical actin, a process dependent on ROCK. MMPs and Rac are not required for amoeboid three-dimensional migration. Mesenchymal cells produce MMPs that are used to digest a path through the matrix for cell movement, but cell contractility is not required.

blebs is restricted by subsequent actin polymerization. The transport of myosin to the region followed by Rho-ROCK contraction causes the retraction of blebs [86]. Although physiologically relevant in cell migration, the function of blebs is not very clear and will probably be elucidated when more is known about how blebs are formed and inhibited.

When migration of neural crest cells was first studied in vitro, it was noted that the initial movements involved vigorous surface blebbing that later resolved into a more rapid mesenchymal mode of locomotion. In vivo, blebbing migration is less evidenced but it has been described by Trinkaus in the epiboly movement in early development of the fish *Fundulus* [87].

Leukocytes demonstrate a version of amoeboid migration that differs from the blebbing, contractility-focused movement of cancer cells. Cell contraction is used to propel the nucleus forward but when the mechanism is impaired through myosin inhibition, the cells continue to navigate through collagen matrices, albeit at slower rates. When the collagen gel concentration is lowered to increase the matrix pore size, the myosin-inhibited cells are able reach the same instantaneous velocity peak value as control cells. In contrast, the actin inhibitor latrunculin B, which interferes with the formation of cell protrusions, significantly reduces the speed of movement irrespective of matrix concentrations. This suggests that the primary mechanism

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that determines migration speed is protrusive movement, but under restrictive conditions, in which the cells are unable to push through collagen by protrusive forces alone, cell contraction supplements migration [88]. Furthermore, this movement occurs independently of integrins in three-dimensional gels – this again differs from mesenchymal migration of fibroblasts and mesenchymal cancer cells that depend on protrusion-mediated mechanisms. In leukocytes, the cell protrusions project forward without exerting pulling forces on the matrix, and the cell contractions at the rear cell body occur in irregular patterns. During cell protrusion, the cell body is passively pulled forward and migration appears to consist of phases of protrusion and contraction that are temporally and spatially unsychronized [88].

MESENCHYMAL AND AMOEBOID MIGRATION AND HETEROGENEITY OF CANCER CELLS

During EMT, autocrine signals in cells can cause chemokinetic behavior (increased random movement), resulting in the movement of tumor cells away from the primary tumor [89]. Once in the circulation, tumor cells can disperse to specific organs, a process known as *homing*, in which chemotaxis (directional movement) plays an important role [90]. *Chemokinesis* is defined as cell motility in the presence of globally distributed soluble factors involving a change in speed or in the frequency or magnitude of turning behavior. *Chemotaxis* is the motility of cells or organisms in which the direction of movement is determined by the gradient of diffusible factors. Kohn et al. [91] have shown that cancer cells demonstrate both chemotaxis and chemokinesis in response to growth factors.

Cell migration is generally evaluated using two methods. The first uses Boyden chambers that allow endpoint assessment of motility, and the second uses live-cell imaging to record the dynamic movement of cells over time [92–95]. In Boyden chambers, cells are seeded into a top well separated by a porous membrane from a bottom well, which contains growth factors. A gradient of growth factors is established across the membrane that stimulates cell migration through the pores from the top to the bottom well (chemotaxis). The pore size is set to require active migration (8–12 μm). When there is a lack of a gradient across the membrane – for example, when growth factors occur in equal concentrations – the migration of cells into the lower chamber is by chemokinetic means.

In heterogenous cancer cells, two subpopulations demonstrating different migration properties were isolated using Boyden chambers set up for either chemotaxis (with a gradient) or chemokinesis (without a gradient) [82]. Cells that migrated into the lower chambers were collected and propagated. When tested for migration, interestingly, cells isolated under chemokinetic conditions (KINE cells) demonstrated both chemokinetic and chemotactic abilities to the same efficiency as the original cell population. The cells isolated under chemotactic conditions (Con cells) were only chemotactic and not chemokinetic. In invasion assays, KINE cells were found to be significantly more invasive than Con cells.

The properties of the subpopulations were further characterised using live-cell imaging assays. When globally stimulated with serum, the KINE cells moved randomly, whereas the Con cells moved more persistently in one direction. These observations are consistent with ideas that random-moving cells are less polarized and that directionally moving cells have a more polarized front-to-back architecture. To understand the polarity of the cell in terms of the relationship between the internal actin cytoarchitecture and the external adhesion sites, co-staining experiments for actin and for adhesion sites using phalloidin conjugates and paxillin antibodies were performed. Actin staining in the KINE cells demonstrated a more rounded morphology, with extensive membrane ruffling at the edge of cells with few stress fibers. Con cells appeared more polarized, with distinctive front and trailing ends. Adhesion sites were not prominent in KINE cells and had the appearance of more transient focal complexes that occur inside the lamellipodium. Con cells, however, were anchored by focal adhesions at the edge of the cells; these adhesion sites were also found where actin stress fibers terminate (Figure 11.4).

The results collectively suggest that the two subpopulations of lung cancer cells known to be chemokinetic and chemotactic also, respectively, demonstrate amoeboid- and mesenchymal-like characteristics in terms of cell polarity, adhesion, morphology, and two-dimensional migration (Figure 11.4). For the first time, data from two different methods of studying cell migration – Boyden chambers and live-cell imaging – can be reconciled. More important, this recognition of the two migration modes has provided important insight into how the variable nature of cells might impact on the weight given to the different methods of study.

There are also important implications in terms of cancer etiology and treatment. For example, in heterogenous populations, treatment with MMP inhibitors will render mesenchymal cells more amoeboid-like in their behavior. Inhibition with ROCK inhibitors will block amoeboid cell migration but not mesenchymal migration. For most tumors, the cellular composition is heterogeneous. This factor is important when selecting the appropriate treatment regime to counter migration and metastasis, and suggest that we need to inhibit both modes of migration simultaneously.

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CURRENT STUDY SYSTEMS FOR EMT AND MIGRATION

Neural Crest Migration

The molecular control of neural crest EMT and migration was initiated when it was demonstrated that many of the principal in vivo features were replicated in vitro in two-dimensional cultures (methodological review by [96]). From these cultures, it was found that EMT and cell locomotion could be triggered by transient cadherin inactivation and also by cytoskeletal manipulation via inhibition of aPKC or Rho and ROCK. The dependency of migration paths on particular adhesively favorable substrates, especially the ECM molecule fibronectin, was also demonstrated. A battery of integrin receptors mediated this, and the ECM adhesive repertoire was seen to be diverse and complex, leading to the idea that the cells were equipped to make sophisticated migratory in response to a variegated ECM microenvironment. The "stripes" assay was developed to test this multisubstrate choice paradigm. In addition, the converse guidance mechanism of repulsion via exclusion zones was realized as a plank now underlying all developmental cell and axon migration studies, and the first ECM repulsive molecule was identified. The driving of locomotion by cell– cell contact (the obverse of "contact inhibition of locomotion") was also described for neural crest cells. Simultaneously, the growth factor signaling and transcription factor expressions that make a neuroepithelial cell into a neural crest cell, determine its EMT, and guide its migration have been revealed. Many of these observations in vitro have now been confirmed in vivo with modern imaging systems and by molecular genetic perturbation techniques (e.g. [97]), and the complexity of molecular controls is being revealed in vivo (see [98, 99]).

The 13762NF System

To examine the metastasis mechanism of amoeboid and mesenchymal cells in culture, we and others have used cells lines that have been isolated from a primary 13762NF mammary adenocarinoma (MTC, mesenchymal) and its lung metastasis (MTLn3, amoeboid) in the same animal [100]. MTLn3 cells retain a high metastatic potential, whereas MTC cells have low metastatic potential. When injected into the mammary fat pads of Fisher 344 rats, MTLn3 cells produced metastases in all axillary lymph nodes and the lungs within four weeks, whereas MTC injection showed metastasis after five weeks in the ipsilateral lymph nodes [101]. The metastatic differential is not caused by the doubling time, as both cell lines have identical doubling time of fourteen hours in culture. The differences in metastatic levels are more likely the result of the different expression levels of EGF receptors. MTLn3 cells express 55,000 receptors per cell, whereas MTC cells do not express EGF receptors [102]. MTC cells engineered to express the same number of (human) EGF receptors as in MTLn3 cells were more chemotactic and showed a higher lung-colonizing ability than parental MTC cells [103]. Thus, amoeboid MTLn3 cells and mesenchymal MTC cells derived from the same primary tumor exhibit very different behaviors in term of metastatic potential and represent an important study model both in vitro and in vivo. The epithelial/mesenchymal status of these cells has not been reported and is under study in our laboratories.

The PMC42 Model

We have developed a novel model system for EGF-induced EMT studies in the human breast cancer cell line PMC42 [26, 63, 104, 105]. Parental PMC42 cells were shown to have stem-like qualities when first characterized [106–109]. They appear mesenchymal (100% vimentin-positive) and respond to EGF with more extreme EMT [26]. An epithelial subline developed by Leigh Ackland of Deakin University in Melbourne forms acini-like structures in three-dimensional Matrigel™ culture; these structures produce milk proteins in response to lactogenic hormone [110] and elaborate myoepithelial markers in peripheral cells when grown as three-dimensional clusters in Matrigel [104, 105]. Stimulation of PMC42-LA cells with EGF also leads to EMT marker expression [26], and three-dimensional Matrigel cultures of PMC42-LA show increased expression of these markers when treated with factors selectively secreted by breast carcinoma-associated fibroblasts over normal mammary fibroblasts [104]. Thus, the PMC42 system provides a spectrum of EMT progression stages on a background of BCSC behavior. We have examined the PMC42 parental cells and found the presence of mesenchymal- and amoeboid-like subpopulations, the latter being in the minority, as expected [16, 26]. Both morphological types invaded efficiently into a three-dimensional collagen matrix, albeit at faster rates for the rounded (ameboid) cells. Within the same experimental culture, the mesenchymal-like and amoeboid cells invaded in a collective and individual manner, respectively (unpublished data).

The MDA-MB-468 Model

MDA-MB-468 cells have a "Basal A" phenotype, suggesting mixed luminal/basal attributes [34], and were recently reported to undergo EGF-regulated EMT [111]. Although predominantly epithelial and E-cadherin-positive [35], a low percentage of cells in culture show vimentin positivity (∼5%), and the cells display intermediate invasiveness [37, 38]. Spontaneous

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lung micrometastases have been reported from MDA-MB-468 (Figure 11.1 and [112]), and GFP-tagged MDA-MB-468 cells isolated from a lung micrometastasis (468LN cells) showed evidence of EMT (spindle-like morphology, increased growth) [112], although their in vivo morphology as primary tumors resemble the parental MDA-468-GFP cells [112]. DNA methylation changes in genes related to EMT and cell migration were recently demonstrated in the 468LN cells [113].

The TSU-Pr1/T24 Model

We have developed an increasingly metastatic bladder cancer progression series using the human TSU-Pr1 (T24) bladder carcinoma cell line. TSU-Pr1 sublines were selected for enhanced bone metastases following intracardiac inoculation and have been previously described [61]. There is a significant increase in the formation of metastatic deposits in a number of tissues from the parental cell line (TSU-Pr1) to TSU-Pr1-B1 (B1) through to the most aggressive cell line TSU-Pr1-B2 (B2). Increased metastasis following systemic inoculation was associated with both phenotypic (decreased in vitro migration, invasion, colony formation) and molecular (increased epithelial cytokeratins, cadherins and membrane-associated β-catenin, decreased vimentin, actin cytoskeleton) – hallmarks of a MET.

CONCLUSION

In early work following the identification of EMT as a transforming event in carcinogenesis, EMT was studied and described as an endpoint function. As we probed further, it has become apparent that the transitions are neither as complete nor as permanent as first thought. During development, for example, examples abound of fluidity in transitional states that are part of a cell's normal repertoire of responses to its microenvironment.

In cancer biology, what is currently deemed as unusual, such as the hybrid cells exhibiting both epithelial and mesenchymal markers, is perhaps quite the norm. Heterogeneity in cancer cell populations in vivo and in vitro is a reality that can be partly explained by the presence of cancer stem cells, the penultimate super cells that continually produce a variety of cell types ranging from epithelial- to mesenchymal- and amoeboid-like. When transplanted from two-dimensional to three-dimensional environments or in animal models, these cells can maintain their respective states but under some circumstances, they switch from mesenchymal to epithelial (MET) or from mesenchymal- to amoeboid-like (MAT). During epithelial sheet migration or cohort migration in three-dimensional matrices, the cells at the leading edge adopt mesenchymal-like characteristics, whereas the attached cells trailing behind remain epithelial-like.

What these paradigms mean to cancer development and treatment is a new appreciation of the chameleonic nature of cancer cells that will drive the derivation of new ways to target this plasticity. The mechanisms facilitating progression from benign to invasive, and finally to metastatic carcinoma, remain largely elusive. A greater understanding of these mechanisms holds much therapeutic and diagnostic promise

ACKNOWLEDGMENTS

The authors gratefully acknowledge grant support from the Australian Research Council (DP0881012), the National Health and Medical Research Council (Australia; EWT #502622 and DFN #436971), Cancer Council Victoria (#509295), and the United States Department of Defense (BC084667). EDW is the recipient of an Australian National Health and Medical Research Biomedical Career Development Award (#519539). SF is supported by an NHMRC grant (#402510)-funded scholarship and AT by an Australian Postgraduate Award from the University of Melbourne.

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A Functional Genomic Screen for Tumorigenicity and Epithelial-Mesenchymal Transition

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Epithelial mesenchymal transition (EMT) has long been associated with breast cancer cell invasiveness and evidence of EMT processes in clinical samples is growing rapidly.

We hypothesize that epithelial – mesenchymal phenotypic attributes affect the capacity of single cells to establish a macroscopically detectable cancer mass, and thus play an etiological role in tumorigenicity, invasion, metastasis and recovery after seemingly effective chemotherapy of breast cancer cells.

Prior to transduction with the whole genome shRNAmir library, a boutique library comprising 4,462 shRNAmir constructs targeting 1,860 markers and mediators of EMT, metastasis, migration, and breast cancer stem cells has been transduced into the EMT-capable PMC42-LA cell line both as a total pool and as ten smaller sub-pools.

These cells undergo epidermal growth factor-induced EMT and show breast cancer stem cell attributes, but do not grow in severe combined immuno-deficient mice when implanted orthotopically or introduced intra-cardially.

The presence of a small but statistically significant proportion of colonies exhibiting markedly mesenchymal features has been observed in the transduced pools, validating the use of this boutique library pool to directly assess whether PMC42-LA tumors can be grown using pools containing a proportion of more mesenchymal cells as an inoculum prior to assessing the whole genome shRNAmir library.

These boutique shRNAmir transduced pools of PMC42-LA cells have been implanted orthotopically and introduced intra-cardially, enabling an assessment of whether tumorigenicity has been impacted upon.